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Nonviral Vectors for Gene Therapy

Lipid- and Polymer-based Gene Transfer

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DEDICATION

We dedicate this book to Professor Feng Liu, who was murdered on July 24, 2014, for his contribution in establishing the procedure of hydrodynamic gene delivery, the most effective and simplest nonviral method of hepatic gene transfer *in vivo* developed so far.

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CHAPTER ONE

Nonviral Vectors: We Have Come a Long Way

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Contents

Abstract

Gene therapy, once thought to be the future of medicine, has reached the beginning stages of exponential growth. Many types of diseases are now being studied and treated in clinical trials through various gene delivery vectors. It appears that the future is here, and gene therapy is just beginning to revolutionize the way patients are treated. However, as promising as these ongoing treatments and clinical trials are, there are many more barriers and challenges that need to be addressed and understood in order to continue this positive growth. Our knowledge of these challenging factors such as gene uptake and expression should be expanded in order to improve existing delivery systems. This chapter will provide a brief overview on recent advances in the field of nonviral vectors for gene therapy as well as point out some novel vectors that have assisted in the extraordinary growth of nonviral gene therapy as we know it today.

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1. INTRODUCTION

The past several decades have shown immense growth in the knowledge of the ability to create and improve nonviral vectors for the delivery of genetic material. This genetic material has great promise as a therapeutic agent against numerous aliments including genetic disorders, chronic and acute diseases, and cancer. Within this field of nonviral vectors, we have produced promising physical methods and chemical vectors for gene delivery consisting of electroporation techniques, cationic lipids, cationic polymers, hybrid lipid polymers, as well as many others. An increased understanding of the field has catalyzed efficiency to new levels in which delivery of plasmid DNA or oligonucleotide into cells can be well characterized and has yielded promising results in preclinical and clinical trials. These vectors have shown to be a promising alternative to viral vectors due to their safety, adaptability, and efficiency in large-scale production. Nonviral vectors have demonstrated their potential to be the next delivery systems of genetic material. They have been shown to exhibit cell specificity through addition of targeting ligands, minimal immune toxicities through addition of inflammatory suppressor molecules, as well as sufficient genetic material release into the cytoplasm of the cell through endosomal destabilization via proton sponge effect or other mechanisms. However, even with these strides, the field of nonviral gene therapy has many areas that need to be addressed, particularly in gene release, nuclear uptake, and expression, which are lagging behind viral vector capabilities. With each vector comes advantages and disadvantages, which will be addressed throughout Part I and Part II of this book.

2. CHEMICAL METHODS

The chemical methods which deliver genetic material via a vector consisting of cationic lipids (lipoplex), cationic polymers (polyplex), or lipid-polymer hybrids (lipopolyplex) have shown promise. These vectors are being used as a systemic approach to delivering genetic material. Therefore, many challenges need to be addressed in order to improve and generate ideal nonviral vectors. These vectors must overcome barriers which consist of extracellular stability, specific cell targeting, internalization, endosomal escape, nucleotide release, nuclear envelope entry, and genome integration (Figure 1.1) (Hu, Haynes, Wang, Liu, & Huang, 2013). These first few

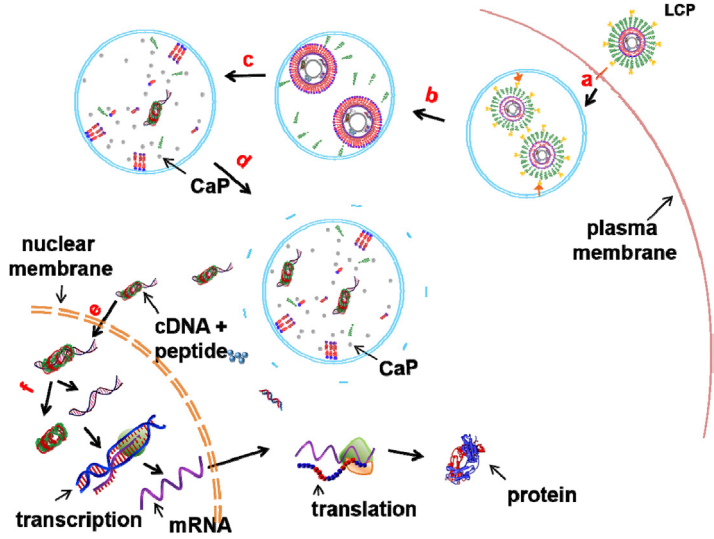

Figure 1.1 ***Proposed mechanism for intracellular delivery of DNA by lipid calcium phosphate (LCP).*** Stepwise scheme for nonviral acid-sensitive vector (LCP), in which (a) the vector is internalized through receptor-mediated endocytosis, (b) PEG is shed from the vector, (c,d) vector and endosome further destabilized as endosome's pH decreases and releases the DNA–peptide complex into the cytoplasm. The DNA–peptide complex enters the nucleus through the nuclear pore, where it dissociates and releases free DNA, which is transcribed to mRNA, migrates to the cytoplasm to be translated, and results in desired protein synthesis (Hu et al., 2013). *Original figure was prepared by Bethany DiPrete.* (See the color plate.)

barriers mentioned seem to have been accomplished to a reasonable level. Multiple vectors have become efficient at achieving long circulation half-life with stable carrier molecules and the addition of hydrophilic moieties such as polyethylene glycol (PEG). The improved cell specificity and internalization with the conjugation of targeting ligands, as well as endosomal escape through the proton sponge effect, have also been achieved with moderate success. By overcoming these initial barriers and being able to deliver genetic material into the cytoplasm of the diseased cell, numerous oligonucleotides, mainly siRNA, are reaching new levels in clinical trials. However, in order to truly reach clinical efficiency in DNA delivery, we must improve intracellular nucleotide release, nuclear entry, and genome integration.

2.1 Cationic Lipid-Based Nanoparticles (Lipoplex)

Cationic lipid-based gene delivery (lipofection) was first published by Felgner's group in the late 1980s (Felgner et al., 1987). It has become

the most studied and popular of all nonviral gene delivery methods and is discussed further in part I, chapters 2, 3, 4, and 7. The basis for using cationic lipids as a delivery system for negatively charged DNA is that the positively charged hydrophilic head group can condense with the DNA while the hydrophobic tail can form micellar or bilayer structures around the DNA. This complexation of lipids around the DNA has been termed a lipoplex and yields DNA protection against nucleases. There are numerous lipid structures that have been tested in order to find optimal lipids to form a lipoplex structure with DNA. The head groups can vary from primary, secondary, and tertiary amines, or quaternary ammonium salts as well as phosphorus, guanidino, arsenic, imidazole, and pyridinium groups. The hydrophobic tails consist of aliphatic chains which can be unsaturated or saturated and are connected to the hydrophilic head by a linker usually consisting of an ester, ether, carbamate, or amide. Cholesterol, as well as other steroids, is usually included in the formulation of these lipoplexes in order to increase the stability and flexibility of these vectors and have been shown to improve transfection *in vivo*. All of these components are critical in formulating promising nonviral gene delivery vectors. Varying these components can drastically change the transfection efficiency as well as improve uptake into the cell and release from the endosome. The electrostatic interaction between the negatively charged cellular membrane and the positively charged lipid head groups is vital in achieving higher levels of cellular uptake. The lipid fusion mechanism in which the positively charged vectors fuse with the cellular membrane ultimately resulting in cellular uptake of genetic material is promoted by vectors with increased flexibility as well as neutral or helper lipids (colipids) that can assist in this fusion with the cellular membrane (Li & Szoka, 2007). The fusogenic properties which facilitate cellular uptake are also valuable in the endosomal escape of lipoplexes through membrane destabilization followed by DNA release from the vector into the cytoplasm of the cell. Although the simple early lipoplexes have the capability to deliver genetic material to cells, they have drawbacks which include low transfection, an inability to target specific cells, short half-life, and toxicity due to the positively charged lipids used. Many more details and examples of cationic lipid vectors are discussed in part I, chapters 2, 3, 4, and 7.

To address the short circulation and toxicity issues with cationic lipid vectors, PEG has been introduced to the surface of these vectors in order to shield the positive charge and reduce opsonization from the reticuloendothelial system. The addition of PEG increased circulation time,

allowing more time for these vectors to transfect cells (Harvie, Wong, & Bally, 2000); however, the surface PEG prevents an interaction between the cationic lipoplexes and anionic cell membrane, reducing the overall transfection efficiency. Therefore, in order to increase cellular uptake of these PEGylated lipoplexes, several strategies have been devised. The conjugation of cell-specific targeting ligands to the distal end of PEG, as well as the addition of PEG-lipid conjugates with shorter alkylated chains that can shed off the vector while in circulation over time, have shown promise. The incorporation of chemically sensitive bonds has also improved the shedding of PEG once inside an acidic or reducing environment such as the endosome or cytoplasm (Li & Szoka, 2007).

Prolonged circulation time and decreased toxicity due to surface modification makes targeted gene delivery to cells located in the interstitial regions possible. Improvements in these nonviral cationic lipid vectors have proved to be promising in gene transfer, especially in the field of siRNA delivery. In addition to its applications in systemic delivery, local DNA and siRNA delivery has shown promise with significant efforts in the delivery of genes directly to the respiratory tract for the treatment of cystic fibrosis, as well as to the cornea and retina for treatment of ocular degenerative diseases (Farjo, Skaggs, Quiambao, Cooper, Naash et al., 2006).

Major preclinical and clinical studies have been completed in the field of cationic lipid gene therapy vectors, but in order for these vectors to truly make a large impact on the medical field, several challenges still lay ahead. Cationic lipids carrying unmethylated CpG DNA have been shown to increase inflammatory responses in the patient (Yew et al., 2000). In addition, quickly dividing cells have shown to have short gene expression due to the DNA dilution over dividing daughter cells. Incorporation of the delivered gene into the cell's genome would allow much more efficient and long-lasting expression of the desired gene. Only when these challenges can be overcome will cationic lipid vectors truly revolutionize the field of gene therapy.

2.2 Cationic Polymer-Based Nanoparticles (Polyplex)

Cationic polymer-based nanoparticles, discussed further in part I, chapters 8, 9, and 10, have been an alternative choice to cationic lipids due to their chemical diversity and potential for functionalization through chemical synthesis. Polyplexes have some advantages over lipoplexes including low enzymatic degradation, more stability, and greater manipulation of their physical characteristics. Two of the earliest and most used cationic polymers

are polyethylenimine (PEI) and poly(L-lysine) (PLL). PLL, which contains cationic lysine residues in physiological pH, is a promising polymer due to its capability to condense DNA, as well as its potential to be conjugated to cell-specific targeting ligands. However, PLL has shown many drawbacks due to a permanent positive charge throughout the life of the polymer *in vivo*. Some of these drawbacks include low levels of escape from the endosome due to buffering from the cationic amines, as well as high levels of toxicity. In order to address these issues, PLL polymers have incorporated endosomal escape moieties such as chloroquine and have added PEG in order to reduce the toxicity caused from the cationic charges. PLL has shown great promise in the field of ocular gene therapy. The DNA is condensed with the cationic PLL and delivered to the desired site by direct injection of the particles. The compacted DNA nanoparticles seem to have no limit on plasmid DNA size, and at high concentrations have been shown to be safe and effective in human clinical trials, provoking no immune responses (Farjo et al., 2006).

The polymer PEI consists of a secondary amine which is only protonated at a lower pH which is achieved in the late endosome. This characteristic of PEI is believed to aid in condensation of DNA and endosomal escape through the proposed proton sponge effect. Although these secondary amines seem to play a vital role in gene delivery and expression levels, other studies suggest that the structural properties, degree of branched or linearity, and molecular weight also play a vital role (Wightman et al., 2001). These structural properties may influence the ability of the polymer to deliver the genetic material into the nuclear membrane after endosomal escape. PEI, however, has also been shown to cause high levels of toxicity and therefore, the PEI–PEG block copolymer has been used in order to create a more biocompatible nanoparticle with longer circulation time.

Second-generation polymers are now being introduced into the field of cationic polymers in order to address the drawbacks of PEI and PLL. These new polymers include a poly[(2-dimethylamino)ethyl methacrylate](pDMAEMA), poly-arginine containing proteins, poly(β-amino ester)s, poly lactic-co-glycolic acid (PLGA)-based nanoparticles, carbohydrate-based polymers such as heparin and dextran, and dendrimers (Mintzer & Simanek, 2009). PLGA-based nanoparticles have been recognized as a potential vector to deliver genes. Research shows that PLGA has an improved safety profile compared to high-molecular weight PEIs and liposomes. Polysaccharides and other carbohydrate-based polymers are also attractive due to high stability, biocompatibility, and biodegradability.

These carbohydrate polymers have also been shown to have lower levels of toxicity compared to PEI and PLL. Dendrimers are highly branched spherical structures with a high population of primary, secondary, and tertiary amines. The most common and promising dendrimer with higher levels of transfection is polyamidoamine. It has been shown that the amine groups and the molecular weight greatly impact expression levels. The mechanism in which dendrimers facilitate gene delivery is one such that the primary amine groups enhance DNA cellular uptake by binding DNA, while the more sterically hindered tertiary amine groups promote endosomal escape via the proton sponge effect (Pack, Hoffman, Pun, & Stayton, 2005).

Similarly to cationic lipids, the levels of gene expression from these polymers fall short of the levels expressed after viral gene delivery. However, these cationic lipids and polymers show promise in preclinical and clinical trials and in improving our knowledge and understanding of how to deliver genetic material to the nucleus of the cell. As our understanding of the mechanisms between nanoparticles and the cellular/nuclear uptake of these materials increases, as will the efficiency of the nanoparticles we formulate.

2.3 Hybrid Lipid-Polymer-Based Nanoparticles (Lipopolyplex)

Hybrid nanoparticles usually consist of a polycation-DNA core with an outer layer shell consisting of lipids. The two main groups are lipid–polycation–DNA (LPD) nanoparticles and multilayered nanoparticles, in which the multilayered nanoparticles are formulated through a layering technique in which cationic polymers and DNA are added sequentially. In most vectors a cationic polymer with the ability to condense DNA is crucial. The main challenge in selecting a cationic polymer is the balance of strong yet reversible electrostatic binding which sufficiently condenses with the anionic DNA backbone, but will release the DNA once cellular/nuclear uptake has occurred. The lipids associated with LPDs can be of two categories: LPDI is referred to when cationic lipids are used, while LPDII is used when anionic lipids are incorporated. The use of cationic lipids can have higher degrees of toxicity, but also improve cellular uptake and endosomal release through the hexagonal fusion with the endosomal membrane. The incorporation of PEG with targeting ligands can also be used to decrease toxicity and improve cell-specific targeting.

These hybrid nanoparticles, such as LCP (mc-CR8C) Gal shown in Table 1.1, express high therapeutic levels of luciferase in the liver of mice (Hu et al., 2013). Although hydrodynamic injections result in an expression

Table 1.1 Comparison of improved hepatic luciferase gene expression in various nonviral gene delivery vectors (Hu et al., 2013)

Nonviral vector	Dose (mg DNA/kg)	Luc expression (RLU/mg protein)
Poly(amine-*co*-ester)	0.5 (i.t.)★	$1.5 \star 10^5$
Bifunctional dendrimer	2.5 (i.v.)§	$7.5 \star 10^5$
Ethyl-alkylated polyethylenimine	2.5 (i.v.)	$1.0 \star 10^6$
R8-GALA-MEND	2.5 (i.v.)	$1.3 \star 10^6$
LCP(mc-CR8C)Gal	0.3 (i.v.)	$4.6 \star 10^7$
Hydrodynamic injection	0.3 (i.v.)	$4.8 \star 10^9$

★Intratumoral tissue injection.
§Intravenous Injection.

level 100 times higher than the LCP vector, it is not necessary to achieve these high levels to have therapeutic effects. Many hybrid nanoparticles are discussed in further detail in part I, chapters 5, 6, and 7. The main challenge still to be addressed, is how to maintain these levels of expression for prolonged periods of time. This may be possible through new findings in which the delivery of genome-editing systems such as zinc-finger nuclease, a transcription activator-like effector nuclease, or clustered regularly interspaced short palindromic repeat-associated system and repair template could allow the integration of the desired genetic material into the cellular genome (Gaj, Gersbach, & Barbas, 2013).

3. PHYSICAL METHODS

Physical methods deliver genetic material, such as naked DNA, through transient penetration of the cell membrane. The most studied of these methods include mechanical, electrical, hydrodynamic, ultrasonic, or magnetic force that have shown much promise. These techniques have minimal toxicity, and in some cases, have shown high levels of expression for periods lasting over 19 months in slow-dividing skeletal muscle. However, it is inherent in many cases that these physical techniques require invasive surgery and cause transient damage at the site of treatment. These techniques are briefly described below and will be covered in more detail in part II of this book.

3.1 Mechanical High-Pressure Delivery

Mechanical high-pressure delivery, also referred to as hydrodynamic injection, was first demonstrated in 1999 by Dr. Feng Liu (Liu, Song, Zhang, & Liu, 1999) and Dr. Guofeng Zhang (Zhang, Budker, & Wolff, 1999). Gene

expression in the liver, kidneys, lungs, and heart was demonstrated by rapid injection of a large volume of naked DNA solution into a mouse via the tail vein. The basic idea of hydrodynamic injection involves generating high pressure in a quick burst resulting in the formation of transient pores in the hepatocytes and subsequent diffusion of DNA into the cells. Hydrodynamic injection is considered to be the most efficient nonviral gene transfer method for in vivo gene delivery in mice. Although hydrodynamic injections show high levels of gene expression in small vertebrates, it is clear that this procedure will need significant modifications before advancing to the clinical setting with human patients. This procedure calls for large injection volumes which are deemed too great for human patients, and also causes transient damage to the target tissues. Improvements in this approach replace systemic injections with catheterization of the target tissues, allowing moderate injection volumes and computer-controlled injection rates. This approach has shown promising gene expression in large-animal studies (Suda, Suda, & Liu, 2008). This improved technique could be the next step in introducing hydrodynamic injection-based gene delivery into clinical trials. Hydrodynamic injection is the most studied physical gene delivery method and is discussed further in part II, chapters 1 and 4.

3.2 Electroporation-Mediated Delivery

Electroporation-mediated delivery of genetic material was first applied to *in vivo* models in the early 1990s by Titomirov AV (Titomirov, Sukharev, & Kistanova, 1991). This method is based on the use of applied electric fields to certain tissues in order to alter the cellular permeability. The formation of transient pores allows genetic material to diffuse through the cellular membrane and into the cell. The general procedure includes the injection of DNA into the target tissue, and subsequent electric force is applied allowing the genetic material to enter the cells. This technique seems to be a safer physical method of introducing genetic material to a tissue compared to hydrodynamic injections. Hashida's group used electroporation methods to achieve tissue specificity following a systemic injection in which high levels of targeted gene expression were found only where an electrical field was applied (Sakai, Nishikawa, Thanaketpaisarn, Yamashita, & Hashida, 2005). Electroporation, discussed further in part II, chapters 1 and 3, has shown much promise with high levels of gene expression in specific targeted tissues, but like many physical methods, electroporation comes with some limitations. Placement of these electrodes requires surgery and in some cases, depending on the target organ, can be very difficult and invasive.

3.3 Ultrasound-Mediated Delivery (Sonoporation)

The use of ultrasound waves to disrupt the plasma membrane allowing material into the cell was first demonstrated in the early 1950s (Fry, Wulff, Tucker, & Fry, 1950). The energy of the wave is absorbed by the tissue, creating abnormalities in the cell membrane which allows material access into the cytoplasm of the cell. The incorporation of microbubbles alongside ultrasound gene transfer has vastly improved this method of gene delivery. The microbubbles, which can be targeted to the desired tissue, act by absorbing the ultrasound waves, breaking apart, and releasing nearby shock waves which can cause the cell membrane to form transient pores. The size of the microbubbles and the agents used in forming these bubbles are critical in order to promote high gene expression. The efficiency of sonication-based gene delivery, discussed further in part II, chapters 1 and 2, is dependent on many factors. These factors include the frequency and intensity of the ultrasound wave, the presence of contrast agent, targeting ability of microbubbles, DNA concentration, and the duration of exposure (Bekeredjian, Grayburn, & Shohet, 2005). Due to the safety and capability of targeting internal organs without surgical procedures, as well as the recent research of enhancing the permeability of the blood–brain barrier, ultrasound-mediated delivery has proved to be a less-invasive physical method. Although microbubbles and ultrasound bring improvements to the field of genetic material delivery, there are issues that need to be addressed. The first issue needing to be addressed is the protection of the genetic material against enzymes and shear forces in the body. Low gene expression levels compared to more invasive and extreme techniques such as electroporation and hydrodynamic injection is a drawback as well. Therefore, by better understanding the exact mechanism of action and optimizing the relationship between the microbubble construct and the ultrasound cavitation, this technique may start to see more promising preclinical and clinical results.

3.4 Magnetic-Sensitive Nanoparticles (Magnetofection)

In an attempt to address the transient damage caused by the invasive methods mentioned above (i.e., hydrodynamic injection and electroporation), magnetofection techniques have been introduced. This technique uses the physical method of a magnetic field to direct the deliver of genetic material to the desired target site. The concept involves attaching DNA to a magnetic nanoparticle usually consisting of a biodegradable substance such as iron oxide and coated with cationic polymer such as PEI (Mulens, Morales, & Barber, 2013). These magnetic nanoparticles are then targeted to the tissue

through a magnetic field generated by an external magnet. The magnetic nanoparticles are pulled into the target cells increasing the uptake of DNA. This technique is noninvasive and can precisely target the genetic material to the desired site while increasing gene expression. The drawback to magnetofection is the need to formulate magnetic nanoparticles complexed with naked DNA, as well as the need for strong external magnets.

4. PERSPECTIVES

The field of nonviral vectors has improved dramatically, gaining ground on the level of expression from viral gene delivery, while also addressing the safety issues that are analogous with these viral vectors. Nonviral vectors over the recent years have proved themselves successful in vivo results that generate therapeutically beneficial levels of expression. Although the transfection efficiency for these nonviral approaches is still well below that of the highly efficient viral vectors; it may not be necessary to achieve these high levels, as long as prolonged expression can be achieved. Further improvements to increase the prolonged expression (part II, chapters 5, 6, and 7) and reduce the toxicity of nonviral vectors (part I, chapter 12) will need to be addressed. In order to meet these needs, our knowledge and understanding of the mechanism of action of nonviral vectors as well as how viral genetic material can be preserved and expressed more efficiently must be improved. Understanding the viral pathway and incorporating the necessary material into a nonviral vector may be the necessary steps needed to successfully achieve a clinically revolutionary gene delivery system.

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CHAPTER TWO

Lipid Nanoparticles for Gene Delivery

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Contents

Abstract

Nonviral vectors which offer a safer and versatile alternative to viral vectors have been developed to overcome problems caused by viral carriers. However, their transfection efficacy or level of expression is substantially lower than viral vectors. Among various nonviral gene vectors, lipid nanoparticles are an ideal platform for the incorporation of safety and efficacy into a single delivery system. In this chapter, we highlight current lipidic vectors that have been developed for gene therapy of tumors and other diseases. The pharmacokinetic, toxic behaviors and clinic trials of some successful lipids particles are also presented.

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1. INTRODUCTION

Lipid nanoparticles (LNPs) have been developed and used extensively as nonviral (or synthetic) vectors to treat genetic and acquired disorders in gene therapy. LNPs are safer than viral vectors due to the absence of immunogenic viral proteins. LNPs have shown robust capability to condense and deliver various nuclei acid molecules ranging in size from several nucleotides (RNA) to several million nucleotides (chromosomes) to cells (Figure 2.1). LNPs are also easy to scale up due to established construction protocols and can be easily modified by the incorporation of targeting ligands. In general, there are three major ways to develop lipidic vectors for suitable gene transfection. The first approach is to screen libraries of lipids to select the most effective structure and biocompatible material for various applications. For example, in the study by Anderson et al., numerous lipids of different structures from the lipid library have been successfully selected and developed to improve the therapeutic efficacy for

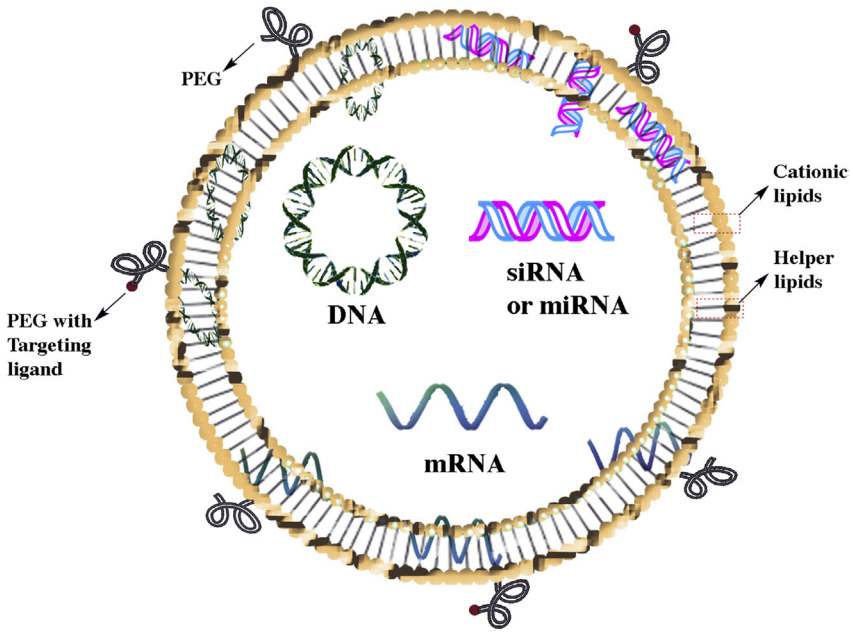

Figure 2.1 Scheme of a lipid nanoparticle (LNP) formed by lipids (yellow), helper lipids (brown), and polyethylene glycol (PEG). Lipids condense and stabilize nucleic acids, which promote the stabilization of LNP. (See the color plate.)

the treatment of various acute and chronic diseases (Chen et al., 2012; Dong et al., 2014; Whitehead et al., 2014). More details are described in the following chapter of this book by Anderson et al. A second approach is to modify current existing lipid materials to enhance the therapeutic efficacy. Some of them have emerged as promising approaches in clinical trials (Tabernero et al., 2013). A third approach is to develop the new materials to deliver genetic material to the target cells (Koynova & Tenchov, 2011). The barriers of gene expression will be briefly described. Several novel lipids and strategies for the improved delivery of nucleic acids are reviewed with an emphasis on the methods of overcoming the limitations caused by the barriers. In addition, we highlight applications of LNP gene therapy in several diseases. Furthermore, the latest studies of pharmacokinetics, biodistribution, and toxicity of LNP gene therapy will be included. In the end, promising clinical studies of LNP-based gene therapy will be discussed.

2. RATIONAL DESIGNS TO OVERCOME EXTRACELLULAR AND INTRACELLULAR BARRIERS

Many disorders, such as cancer, are disseminated and widespread throughout the body, thus intravenous injection of agents is the most common, but also the most complex, route in gene therapy. From the moment of injection until the agent reaches targeted cells, genetic material encounters extracellular and intracellular barriers that affect the therapeutic results. First, naked RNAs or DNAs are unstable under physiological conditions, resulting in enzymatic degradation by endogenous nucleases and clearance by the reticuloendothelial system (RES). Second, RNAs or DNAs are anionic hydrophilic polymers that are not favorable for uptake by cells, which are also anionic at the surface. Third, the off-target effect of genes will lead to unwanted toxicities in normal tissues. Furthermore, immune stimulation upon injection hinders further development of new gene therapies. The success of gene therapy depends largely on the development of a vehicle or vector that can efficiently and effectively deliver genetic material to target cells and obtain sufficient levels of gene expression in vivo with minimal toxicity. Virus-derived vectors for gene therapy are efficient in gene delivery and transfer, but safety issues limit the use of viral vectors in gene therapy. To date, the rational designs of nonviral vectors have been focused on overcoming the extracellular and intracellular barriers in the delivery of genetic material to targeted cells.

2.1 Extracellular Barriers

Once exogenous genes enter the human biological system, they are recognized by the RES as foreign pathogens and cleared from blood circulation before having the chance to encounter target cells within or outside the vascular system (Mastrobattista, van der Aa, Hennink, & Crommelin, 2006). It has been reported that the half-life of naked DNA in the blood stream is around several minutes (Kawabata, Takakura, & Hashida, 1995). Upon injection, DNA was rapidly degraded by enzymes and eliminated from plasma due to extensive uptake by the liver (Kawabata et al., 1995). Chemical modification and a proper delivery method can reduce uptake by RES and protect nucleic acids from degradation by ubiquitous nucleases, which increase stability and efficacy of gene therapy.

Many efforts have been made to increase the stability and half-life of liposomes in the body by incorporation of helper components. For example, Damen (Damen, Regts, & Scherphof, 1981) and Semple (Semple, Chonn, & Cullis, 1996) incorporated cholesterol into the membrane to reduce the mobility of phospholipid molecules and increase packing of phospholipid.

Coating the liposome with polyethylene glycol (PEG), or PEGylation, is typically the method used to protect nanoparticles from the immune system and escape RES uptake (Jokerst, Lobovkina, Zare, & Gambhir, 2011). Since 1990s, PEGylation has been widely used to stabilize liposomes and their payloads through physical, chemical, and biological mechanisms. Detergent-like PEG lipids (e.g., PEG-DSPE) can enter the liposome to form a hydrated layer and steric barrier on the liposome surface. Based on the degree of PEGylation, the surface layer can be generally divided into two types: brush-like and mushroom-like layers. For PEG-DSPE-stabilized liposomes, PEG will take on the mushroom conformation at a low degree of PEGylation (usually less than 5 mol%) and will shift to brush conformation as the content of PEG-DSPE is increased past a certain level (Guo & Huang, 2011). It has been shown that increased PEGylation leads to a significant increase in the circulation half-life of liposomes (Huang & Liu, 2011; Li & Huang, 2010). However, due to the detergent-like property of PEG-DSPE, the brush layer with high PEGylation degree is not stable. Li and Huang discovered that PEGylated liposome–polycation–DNA (LPD) nanoparticles overcome this issue (Li & Huang, 2010). The LPD nanoparticle is stabilized by electrostatic interactions within the negatively charged nucleic acid–protamine complex core and positively charged lipid bilayer. This core-surface type of liposome was able to support the bilayer and tolerate a high level of PEG-DSPE (10 mol%) with a relatively dense PEG

brush structure on the surface. Most importantly, these liposomes were not taken up by the liver Kupffer cells (Li & Huang, 2009). Furthermore, modification of sheddable PEG with tumor-specific ligands or pH-sensitive linkers has extended the use of LNPs in gene therapy. However, upon multiple injections, PEGylated LNP loses its ability to circulate for long periods in the bloodstream, a phenomenon known as accelerated blood clearance (ABC) (Dams et al., 2000; Gomes-da-Silva et al., 2012). The mechanism of ABC is associated with activation of anti-PEG-specific IgM after the first dose of PEGylated liposome (Ishida et al., 2006).

Recently, Liu, Hu, and Huang (2014) used the lipid bilayer core structure of the lipid–calcium–phosphate (LCP) NPs to examine the effects of the density of PEG and the incorporation of various lipids onto the surface in vivo. In their study, they demonstrated that delivery to hepatocytes was dependent on both the concentration of PEG and the surface lipids. Moreover, LCP NPs could be directed from hepatocytes to Kupffer cells by decreasing PEG concentration on the particle surface. Positively charged lipid 1,2-dioleoyl-3-trimethylammonium-propane exhibited higher accumulation in the hepatocytes than LCP NPs with neutral lipid dioleoylphosphatidylcholine.

As a systemic delivery carrier, LNPs must be stable enough to remain in circulation for an extended period and accumulate at disease sites via the enhanced permeability and retention (EPR) effect. In addition to working with lipid vectors, recent studies have also found that chemically modified nucleic acids can increase stability by altering the physicochemical properties. For instance, without significant loss of RNA interference activity, Czauderna et al. showed that chemical modification of siRNA at different positions can stabilize siRNA against serum-derived nucleases and prolong the circulation time in the blood (Czauderna et al., 2003).

2.2 Intracellular Barriers

It has been reported that although >95% of cells in culture typically internalize vectors, only a small fraction, typically <50%, express the transgene (Mark, 2003). Following internalization, gene delivery vectors are challenged by intracellular barriers, including endosome entrapment, lysosomal degradation, nucleic acid unpacking from vectors, translocation across the nuclear membrane (for DNA), release at the cytoplasm (for RNA), and so on. Successful gene therapy depends upon the ability of the vector to deliver the nucleic acids to the target sites inside of the cells in order to obtain sufficient levels of gene expression. The relative contribution of distinct

endocytic pathways, including clathrin- and caveolae-mediated endocytosis and/or macropinocytosis, is not yet well defined. Escape of DNA/RNA from endosomal compartments is thought to represent a major obstacle. LNPs have shown the unique ability to deliver nucleic acids by endosomal escape. Initially, Szoka et al. proposed that anionic phospholipids could displace cationic lipids from plasmids, thus assisting the release of plasmid following uptake of the complex into cells (Xu & Szoka, 1996; Zelphati & Szoka, 1996). It is also suggested that cationic lipids form ion pairs with anionic lipids within the endosome membrane leading to disruption of the endosomal membrane following uptake of nucleic acid–cationic lipid complexes into cells. This facilitates cytoplasmic release of the plasmid or oligonucleotide (Hafez, Maurer, & Cullis, 2001). In addition, Cullis et al. proposed that mixtures of cationic lipids and anionic phospholipids preferentially adopt the inverted hexagonal (H_{II}) phase, therefore facilitating escape of the plasmid from the endosome into the cytoplasm (Cullis, Hope, & Tilcock, 1986; Hafez et al., 2001). Significant intracellular hurdles beyond endosomal escape include the limited nuclear entry (Brunner et al., 2000; Dean, Strong, & Zimmer, 2005) and inefficient intranuclear release of plasmid for transcription (Hama et al., 2006). The transfection efficiency of nanoparticles is also related to the cell cycle and is enhanced by mitotic activity. For instance, Brunner's study showed that the high transfection close to the M phase is facilitated perhaps by nuclear membrane breakdown at this phase (Brunner et al., 2000). Hama et al. compared the intracellular trafficking and nuclear transcription between adenoviral and lipoplex (lipofectamine plus). In their observation, although lipoplex system has higher cellular uptake than that of adenoviral vector, the nuclear transfer efficiency of lipoplex is found to be lower than the adenoviral one, suggesting that the difference in transfection efficiency principally arises from differences in nuclear transcription efficiency and not from a difference in intracellular trafficking (Hama et al., 2006).

3. CURRENT LIPIDIC VECTORS FOR GENE DELIVERY

3.1 Cationic Lipids

Cationic lipids were introduced as carriers for delivery of nucleic acids for gene therapy over two decades ago (Malone, Felgner, & Verma, 1989; Schroeder, Levins, Cortez, Langer, & Anderson, 2010). They are still the major carriers for gene delivery, because they can be easily synthesized and extensively facilitated by modifying each of their constituent domains.

Cationic lipids can be used as vectors to condense and deliver anionic nucleic acids through electrostatic interactions. These nanostructured complexes, called "lipoplexes," have shown to be extremely useful vehicles in gene therapy. By modulating the ratio of cationic lipids and nucleic acids, the excess cationic coating was able to facilitate the binding of vectors with negatively charged cell surfaces, and furthermore interruption with endosomal membrane to help cytoplasmic delivery of nucleic acids. However, lipoplex suspensions are known to be unstable in aqueous suspension for long-term storage, especially with respect to hydrolysis and size stability (Fehring et al., 2014). DNA can be encapsulated in liposomal formulations by thin film, reverse-phase evaporation and asymmetric liposome formation methods (Levine, Pearce, Adil, & Kokkoli, 2013).

It has been demonstrated that the physicochemical properties of cationic lipids significantly limit the cellular uptake and transfection efficiency in gene therapy. In a study by Ross et al, it was found that the size of the lipoplex is a major determinant of in vitro lipofection efficiency (Ross & Hui, 1999). Furthermore, reports from different laboratories have demonstrated that larger liposomes are eliminated from the blood circulation more rapidly than smaller ones (Senior, 1987) and positively charged liposomes have a shorter half-life than neutral or negative ones (Immordino, Dosio, & Cattel, 2006).

Following previous fundamental studies on the structure–activity relationship of cationic lipids, it is well accepted that the polar headgroup, hydrophobic moiety, and linker are three important constituent domains for cationic lipids. While hydrophobic regions, including the length and the degrees of nonsaturation of the alkyl chains, are relatively similar, the structure and component of polar headgroup and linkers are substantially different. The polar hydrophilic headgroup is positively charged, usually through the protonation of one or several amino groups. They can be quaternary ammoniums, amines, amino acids or peptides, guanidiniums, heterocyclic headgroups, and some unusual headgroups (Zhi et al., 2013). The hydrophobic portion of lipid is composed of a steroid or alkyl chains (saturated or unsaturated). The headgroups of cationic lipids exhibiting one or more positive charges can condense negatively charged nucleic acids through electrostatic attraction. This binding force plays an important role in the therapeutic efficacy of gene therapy. On one hand, it has to be strong enough to protect nucleic acids from degradation during circulation and transportation. On the other hand, it also has to be weak enough to allow for timely release of the payload of nucleic acids within target cells.

Semple et al. found that the acid dissociation constant (pKa) of the headgroup and the distance of the charge presented to the lipid bilayer interface are the most important parameters for siRNA delivery in vivo (Semple et al., 2010). Recently, Alabi et al. also supported the observation that they found the pKa of LNPs showed the strongest correlation with biological barriers and gene silencing (Alabi et al., 2013). The presence of a charge on the lipid can lead to toxic side effects and rapid clearance from the circulation. To address this problem, anionic lipids with pKa values of 7 and lower have been synthesized, which have presented lower toxicity and efficient encapsulation of nucleic acids for both in vitro and in vivo activity (Tam, Chen, & Cullis, 2013). More details about LNPs for short interfering RNA delivery are presented in the following chapter of this book by Cullis et al.. When vectors reach the physiological acidic environment of the endosome, the amine should be protonated and become positively charged, associating with the anionic endosomal lipids, inducing an endosomolytic H_{II} (inverted micelle) phase structure. This interaction will induce the destabilization of the endosomal membranes and promote the release of siRNA into the cytosol. Schroeder et al. had shown that molecules containing several amines per head group are able to adhere to the negatively charged siRNA in a better manner than several lipids containing a single positive charge per headgroup (Schroeder et al., 2010).

3.2 Ionizable Lipids and Lipidoids

Ionizable lipids are an advanced delivery platform of gene therapy that can self-assemble into nanoparticles when mixed with polyanionic nucleic acids. Ionizable cationic lipids with modulated pKa values increase nucleic acid payload and enhance the therapeutic efficacy of gene therapy. At formulating step, where there is a low pH condition, ionizable lipids will become positive charged, resulting in high nucleic acids loading. While, upon injection, in physiological environments where the pH is above the pKa of the ionizable lipids, the surface of the LNPs has an almost neutral charge that can evade RES uptake, improve circulation, and reduce toxicity (Tam et al., 2013). However, once nanoparticles are internalized into the endosome, where the pH is lower than the pKa of the lipids, the amino group of the ionizable lipid becomes protonated and associates with the anionic endosomal lipids, which facilitate endosome escape. Recently, two promising ionizable cationic lipids (Figure 2.2), DLin-KC2-DMA (2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane) with a pKa of 6.7, and DLin-MC3-DMA (1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane)

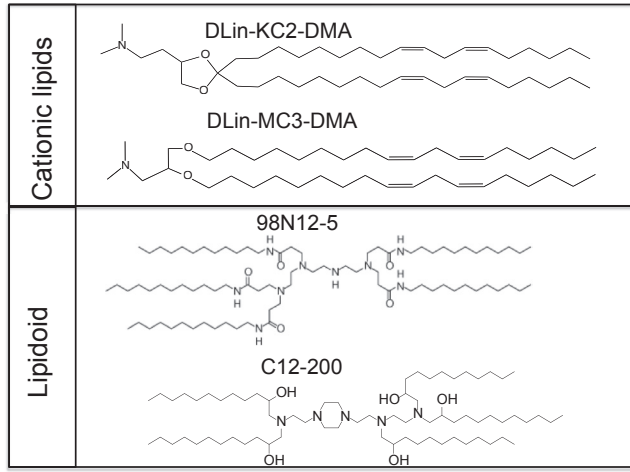

Figure 2.2 A sampling of lipids as nonviral vectors for gene delivery.

with a pKa of 6.4, have been successfully developed to formulate stable nucleic acid lipid particles (SNALPs) (Heyes, Palmer, Bremner, & MacLachlan, 2005; Jayaraman et al., 2012; Semple et al., 2010), which are 100-fold and 1000-fold more potent in silencing hepatic genes in comparison to the previously used lipids (Heyes et al., 2005). Most excitingly, they decreased the half-maximal effective dose (ED_{50}) of the siRNA in rodents from ~0.1 mg/kg to ~0.02 mg/kg and presented excellent silencing activity in rodents as well as nonhuman primates. Recently, Tekmira, a biopharmaceutical company, announced in its website that the third generation lipids successfully integrated to deliver mRNA, and achieved a higher efficacy than DLin-MC3-DMA LNPs, however, the details about this lipid is not yet published.

A new class of lipid-like material, termed lipidoids, which contain tertiary amines, are one of the most innovative and promising nonviral lipid vehicles for RNAi therapeutics (Akinc et al., 2008). They are prepared by conjugating commercially available amines (Figure 2.2.) (Akinc et al., 2009; Love et al., 2010; Sun et al., 2012). Notably, the synthesis reaction for generating a lipidoid library proceeds in the absence of solvent or catalysts, and thereby eliminates the purification or concentration steps (Akinc et al., 2008). Lipidoids and lipids share many of the physicochemical properties that drive the formation of liposomes for gene delivery. However, lipidoids are easy to synthesize and purify and do not require a colipid for efficient DNA delivery. These advantages make high-throughput combinatorial synthesis of lipidoids possible and

allow for rapid in vitro screening of thousands of potential drug delivery candidates (Figure 2.2). By varying the types of amines and the lengths and types (acrylamide/acrylate/epoxide) of tails, Sun et al. were able to build a structurally diverse library (Sun et al., 2012). Lipidoids will be discussed in further detail in the following chapter of this book by Anderson et al.

3.3 Gene-Lipid Conjugates

As known, nucleic acids are rapidly degraded in serum or inside cells and must be protected from nuclease attack. Even though cationic lipids with different functionalities have been used to encapsulate nucleic acids from degradation and enhanced the therapeutic efficacy, several studies have shown that cationic lipids exhibit severe toxicities, resulting in the limitation of further clinical applications (Soenen, Brisson, & De Cuyper, 2009; Yew & Scheule, 2005). The simplest approach to increase nuclease stability is to directly modify the internucleotide phosphate linkage (Behlke, 2008). Instead of providing a carrier for nucleic acids, several studies have reported that nucleic acid conjugation could improve in vivo pharmacokinetic behavior of genetic materials, providing an alternative approach for gene therapy (Chillemi, Greco, Nicoletti, & Sciuto, 2013; Koppelhus, Shiraishi, Zachar, Pankratova, & Nielsen, 2008; Kubo et al., 2013). Conjugating the lipids to the site of nucleic acids without loss of bioactivity is the key step for modification. Replacement of a nonbridging oxygen with sulfur, boron, nitrogen, or methyl groups provides nuclease resistance and has been extensively explored for use in antisense applications (Behlke, 2008). Exogenous siRNA can activate the innate immune system through toll-like receptors (TLR), but introduction of 2′-O-methyl (2′OMe) to nucleotide can inhibit the TLR-associated inflammatory response (Judge, Bola, Lee, & MacLachlan, 2006).

Hydrophobic lipids can also be attached to siRNAs to change the biodistribution, extend circulation time, and facilitate direct cellular uptake (Lorenz, Hadwiger, John, Vornlocher, & Unverzagt, 2004; Soutschek et al., 2004; Wolfrum et al., 2007). For example, cholesterols have been successfully introduced to conjugate to the 3′-terminus of the sense strand of siRNA nucleic acids for systemic delivery via a pyrrolidone linkage (Soutschek et al., 2004). The conjugate (chol-siRNA) exhibited increased cellular transfer efficiency and improved in vivo pharmacokinetic behaviors without a significant loss in silencing ability. Another biocompatible material, α-tocopherol (vitamin E), can be covalently conjugated to the 5′-terminus of the antisense strand of siRNA to achieve a significant

reduction in targeted protein without induction of inflammatory interferon (Nishina et al., 2008). However, chemical modification of siRNA alone often results in renal clearance of intact siRNA without degradation (Behlke, 2008). As such, for future application of chemically modified siRNA, rational design and increased specificity are in great need.

3.4 LNP Functionalization

To enhance targeted delivery, several functional LNPs for gene therapy have been developed recently. With these proof-of-concept systems, functionalized particles efficiently delivered associated nucleic acids to the targeted cells. The first strategy is to modify the nanoparticles with tumor-specific ligand to enhance intracellular uptake. For example, iron-saturated transferrin (Tf) (Huang et al., 2013), folic acid (Hu et al., 2014; Xiang et al., 2013), RGD (Han et al., 2010; Majzoub et al., 2014), and anisamide (Li, Chono, & Huang, 2008) have been widely applied for specific gene delivery.

The rational design of LNPs to escape from endosomal/lysosomal vesicles is another strategy to enhance the efficacy of gene therapy. The extracellular pH of tumor inflammatory tissues is lower than other physiological tissues. Following internalization, most vectors end up in compartments with a lower pH. Endosomes have a pH around 5.5–6.0 and lysosomes about 4.5. Thus, if pH-sensitive functional groups are applied to the LNPs, they may become protonated in the low-pH environment. This would result in lower toxicity and facilitate the delivery of nucleic acids before degradation (Hu et al., 2014). Generally, pH-sensitive lipids contain a tertiary amine instead of a quaternary ammonium group, which results in a cationic charge at an acidic pH and almost neutral at physiological pH (Sato et al., 2012). Moreover, there are some successful pH-sensitive linkers applied to nanoparticles to achieve more specific delivery, for example, diorthoester, orthoester, vinyl ether, phosphoramidate, hydrazine, and beta-thiopropionate (Romberg, Hennink, & Storm, 2008).

Magnetic LNPs are particles that have magnetic cores with a lipid coating that can be functionalized by attaching therapeutic nucleic acids to correct a genetic defect (McBain, Yiu, & Dobson, 2008; Ranjan & Kinnunen, 2012). Biocompatible and biodegradable iron oxide nanoparticles can be used as contrast enhancement agents for magnetic resonance imaging and also act as effective carriers for genes (Jiang, Eltoukhy, Love, Langer, & Anderson, 2013; McBain, Yiu, & Dobson, 2008). Jiang et al. used C14-200 lipidoids and DSPC to coat iron oxide nanoparticles in N-methyl-2-pyrrolidone solvent and showed efficient DNA and siRNA delivery upon

the application of an external magnetic field, with performance exceeding that of Lipofectamine 2000 (Jiang et al., 2013). Kenny et al. developed an MRI-visible gene delivery nanocomplex system comprised of self-assembling mixtures of liposomes, plasmid DNA, and targeting ligands, which specifically enhanced transfection efficiency and allowed real-time in vivo monitoring of the specific tumor tissue (Kenny et al., 2012). In another study, Writer et al. prepared lipid peptide nanocomplexes with Gadolinium-chelated lipid, DNA-binding peptide, and plasmid DNA (Writer et al., 2012). These lipid nanocomplexes can be used for gene delivery and MRI imaging in the brain. LipoMag, a novel LNP developed by Namiki et al., is made of an oleic acid-coated magnetic nanocrystal core and a cationic lipid shell (Namiki et al., 2009). It displayed efficient gene silencing and antitumor efficacy without an adverse immune reaction upon injection in mice bearing gastric tumors.

Microbubble ultrasound contrast agents have the potential to dramatically improve gene therapy treatments by enhancing the delivery of therapeutic nucleic acids to malignant tissues. Ultrasound technology has the ability to improve cell membrane permeability, modulate vascular permeability, and enhance endocytic uptake in cells. In a recent study by Fujii et al., ultrasound-mediated transfection of VEGFR2 shRNA plasmid-bearing microbubbles resulted in knockdown of VEGFR2, leading to an antiangiogenic effect and reduced tumor growth (Fujii et al., 2013). Song et al. explored high-intensity therapeutic ultrasound- and microbubble-mediated gene delivery. Maximum gene expression in treated animals was 700-fold greater than in negative controls (Song, Shen, Chen, Brayman, & Miao, 2011).

4. GENE THERAPY APPLICATIONS

Up until 2014, over 2000 clinical trials, comprised of virtually all types of human disorders, have been conducted or were currently ongoing for gene therapy. The number of clinical trials is still increasing due to the promising opportunity to correct gene disorders.

4.1 Gene Therapy for Cancer

Much attention of today's cancer research is focused on finding missing or defective genes that cause or increase an individual's risk of certain types of cancer. Over 60% of the gene therapy clinical trials conducted have been in the field of cancer (Giacca, 2010). Cancer gene therapy can benefit from

two aspects based on the mechanism of gene medicines. First, gene therapy can directly affect specific genes that cause cancer at the molecular level. Second, gene therapy can prevent cancer by improving the immune system through identifying the susceptibility genes. In other words, LNP-based cancer gene therapy can follow two alternative approaches: eliminate the cancer cells or improve the efficacy of the immune system by recognizing and destroying cancer cells.

As a result of rapid, defective angiogenesis, tumor blood vessels are highly permeable, leading to accumulation of nanoparticles at the tumor site. Furthermore, tumors are characterized by dysfunctional lymphatic drainage that extends the retention of LNPs at the tumor site. This behavior of nanoparticles is called the EPR effect proposed by Dr Maeda (Maeda, 2012; Maeda, Nakamura, & Fang, 2013; Matsumura & Maeda, 1986). However, the leakage of blood vessels in different types of tumors is quite different and limited experimental data from patients on the effectiveness of this mechanism have hindered the development of effective drugs (Prabhakar et al., 2013). Vascular permeability is the key factor involved in the EPR effect in cancer. It is well accepted that vascular endothelial growth factor (VEGF) enhances the vascular permeability of tumor vessels. In a recent study by Zhang, Schwerbrock, Rogers, Kim, and Huang (2013), VEGF siRNA and gemcitabine monophosphate (GMP) were encapsulated into a single cell-specific, targeted LCP nanoparticle formulation, resulting in 30–40% induction of tumor cell apoptosis, eightfold reduction of tumor cell proliferation, and significant decrease of tumor microvessel density. This combination therapy led to improved therapeutic response in comparison to either VEGF siRNA or GMP therapy alone. Recently, first-in-humans trial of an RNAi therapy targeting VEGF and kinesin spindle protein in cancer patients was performed using LNP-formulated siRNA therapy (Tabernero et al., 2013). They detected the drug in tumor biopsies, siRNA-mediated mRNA cleavage in the liver, downregulation of the targeted gene, and antitumor activity. These results presented proof-of-concept for RNAi therapeutics with LNP formulation in humans.

4.2 Gene Therapy in Liver Disease

Liver diseases, including inherited metabolic disorders, chronic viral hepatitis, liver cirrhosis, and primary and metastatic liver cancer constitute a formidable health problem due to their high prevalence and the limitations of current therapies (Domvri et al., 2012; Gonzalez-Aseguinolaza & Prieto, 2011; Prieto et al., 2004). For most of the inherited metabolic liver diseases,

no effective therapy is currently available other than liver transplantation, which is hampered by donor shortage, cost, surgical risks, and long-term immunosuppression. Thus, safer and more efficient therapies are greatly needed. Nonviral carriers for liver gene therapy can fulfill these needs as they are able to delivery gene-based medicines more specifically to the liver with minimized toxicity and immunogenicity. Taking advantage of special membrane receptors located on liver cell membrane, nonviral vectors, especially LNPs, can be modified with targeting moieties and deliver the targeted genes to liver. Several attempts have shown potential success in liver disease. For example, collagen type VI receptor (Du et al., 2007), mannose-6-phosphate receptor (Adrian et al., 2007), and galactose receptor (Mandal, Das, Basu, Chakrabarti, & Das, 2007) have been successfully targeted. Sato et al. designed vitamin A-coupled liposome to deliver siRNA for liver cirrhosis. In their study, only five treatments with the collagen-specific liposomes almost completely resolved liver fibrosis and prolonged survival in rats with otherwise lethal dimethylnitrosamine-induced liver cirrhosis in a dose- and duration-dependent manner (Sato et al., 2008).

5. PHARMACOKINETICS, BIODISTRIBUTION AND TOXICITY OF LNPS

5.1 Pharmacokinetics and Biodistribution Profile of LNPs

It is well known that the systemic delivery of naked DNA or siRNA alone often lead to fast clearance and degradation. A variety of lipid vectors hold the potential to improve gene therapy. As long as the nucleic acids are completely encapsulated in stable vectors, the system could provide protection to the nucleic acids from degradation, and thus, the vector will be able to represent the biodistribution of whole system. One of the key reasons for this success is that LNPs are able to provide better biodistribution and pharmacokinetics profiles of genes in vivo.

In order to study LNPs, the nucleic acids and vehicles were labeled with radioactive isotopes and tracked upon the administration. Replacing ^{1}H or ^{12}C atoms of nucleic acids with radioactive ^{3}H or ^{14}C does not alter the structure of nucleic acid and have the least impact on the pharmacokinetic behavior of nucleic acid itself. However, van de Water et al. reported a head-to-head comparison of ^{3}H- versus ^{111}In-labeled unformatted siRNA, and found that they have different distributions and pharmacokinetics (Christensen et al., 2013; van de Water et al., 2006). Therefore, it is important to choose the right modification of radioactive isotopes to monitor the behavior of nanoparticles in the

body. However, as radioactive compounds are potential health hazards some studies use fluorescence imaging to track the distributions and pharmacokinetic profiles of LNPs in gene therapy. Although fluorescent dyes are relatively safe with low cost, they may not be the best option. For example, Liu et al. recently compared radioactive isotopes and fluorescence imaging using LCP nanoparticles and found that while radioactive isotopes showed the liver and spleen as the major accumulation sites, fluorescence imaging indicated tumor accumulation was predominant. A possible explanation for this difference is that the liver and spleen have strong intrinsic tissue absorption and light scattering which quenches fluorescence (Liu, Tseng, & Huang, 2012).

In general, following systemic injection, positively charged lipid/nucleic acid formulation will bind to various types of serum proteins such as albumin, heparin, lipoprotein, specific opsonins, and others. The binding force is dependent on net charge density and surface morphology of the lipid/plasmid complex (Thierry et al., 1997). Extensive lung accumulation was observed after injection, which may be the result of entrapment of complexes in lung capillaries by the first-passage effect. Lung deposition may also be due to ionic association with the large surface area of the lung endothelia (Mahato et al., 1998). Negatively charged complexes did not show lung accumulation (Ishiwata, Suzuki, Ando, Kikuchi, & Kitagawa, 2000).

5.2 Toxicity of LNPs

The composition of LNPs for the gene therapy can be divided into two parts: nucleic acids and lipids. Thus, safety issues related to LNPs in gene therapy are caused by nucleic acid- and lipid-mediated side effects. The major problem in gene therapy is the off-targeting effect, in which nucleic acids will distribute nonspecifically throughout the whole body. Studies have shown that introduction of dsRNA longer than 29–30 bp into mammalian cells results in a potent activation of interferon response and have precluded its use in RNAi-based therapy (Huang & Liu, 2011). Exogenous siRNA can activate the innate immune system through TLR 7 and 8. Thus, careful design and adequate control are greatly needed for gene therapy if naked nucleic acids are directly administrated into the body.

An off-target side effect of naked nucleic acids can be partially eliminated through incorporation into a lipid formulation conjugated with a targeting moiety. However, some lipids are also immunogenic. Of these lipids, the immunostimulating effects are reported to be stronger in cationic liposomes than anionic or neutral liposomes. Cationic liposomes alone can stimulate antigen-presenting dendritic cells leading to the expression

of co-stimulatory molecules, CD80 and CD86 (Vangasseri et al., 2006). Recently, Omidi et al. used a microarray method to evaluate the toxicogenomics and genotoxic potential in a biological system after cationic lipid-based gene therapy (Omidi, Barar, & Akhtar, 2005) and found that cationic lipid Oligofectamine nanosystems in human alveolar epithelial A549 cells induced significant gene expression changes belonging to the different genomic ontologies such as cell defense and apoptosis pathways (Omidi et al., 2008). The data indicated the importance of safety and immunogenicity examination of new lipids for gene therapy.

6. CLINICAL TRIALS

Academic and industrial researchers have made steady progress in gene therapy since Friedmann et al. first proposed the use of genes for human genetic disease in 1972 (Friedmann & Roblin, 1972). Recently, several therapeutic agents targeting various types of diseases have reached different stages of clinical trials (Table 2.1). Alnylam Pharmaceuticals are developing aggressive LNP therapeutic agents using Enhanced Stabilization Chemistry-GalNAc-conjugate delivery technology. For example, ALN-TTRsc (targeting TTR for treatment of transthyretin-mediated amyloidosis) and ALN-PCS02 (targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) to lower cholesterol for treatment of hypercholesterolemia) are currently used in clinical trials. ALN-TTR02, known as Patisiran recently released clinical data that the treatment of LNPs achieved sustained serum TTR protein knockdown of 96% with a mean TTR knockdown of about 80% (www.alnylam.com). The recent report of 32 participants in Phase I had shown that ALN-PCS treatment resulted in a mean 70% reduction in circulating PCSK9 plasma protein and a mean 40% reduction in low-density lipoprotein (LDL) cholesterol from baseline relative to placebo (Fitzgerald et al., 2014).

SNALP technology from Tekmira Pharmaceuticals, Inc. is one of the most widely used lipid-based nucleic acid delivery approaches for systemic administration in clinical trials. Lipid vesicles encapsulating nucleic acids are formed instantaneously by mixing lipids dissolved in ethanol with an aqueous solution of nucleic acids in a controlled, stepwise manner. Using this method, SNALP encapsulates nucleic acids with high efficiency (95%) in uniform LNPs, which are effective in delivering gene therapeutics especially to hepatocytes. Tekmira's lead oncology product candidate, TKM-PLK1, targets polo-like kinase 1 (PLK1), a protein involved in tumor cell

Table 2.1 Examples of currently evaluated new LNPs for siRNA clinical trial

Therapeutic agent & company	Target	Clinical trial identifier	Disease types	Status
ALN-VSP02; Alnylam	Kinesin spindle protein; vascular endothelial growth factor	NCT00882180 NCT01158079	Solid tumor	Phase 1
ALN-TTR02; Alnylam	Transthyretin	NCT01617967 NCT01960348	Transthyretin-mediated amyloidosis	Phase 2/3
ALN-PCS02; Alnylam	Proprotein convertase subtilisin/kexin type 9	NCT01437059	Hypercholesterolemia	Phase 1
TKM-100201; Tekmira	VP24,VP35, Zaire Ebola L-polymerase	NCT01518881	Ebola virus infection	Phase 1
PRO-040201; Tekmira	ApoB	NCT00927459	Hypercholesterolemia	Phase 1
TKM 080301; Tekmira	Polo-like kinase 1	NCT01262235 NCT01437007	Neuroendocrine tumors; adrenocortical carcinoma	Phase 1/2
siRNA-EphA2-DOPC; M.D. Anderson Cancer Center	Ephrin type-A receptor 2	NCT01591356	Advanced cancers	Phase 1
Atu027; Silence Therapeutics	Protein kinase N3	NCT00938574	Advanced solid tumors	Phase 1

DOPC, dioleoylphosphatidylcholine.

proliferation and a validated oncology target. Tekmira initiated a Phase I/II clinical trial of TKM-PLK1 for patients with gastrointestinal neuroendocrine tumors, adrenocortical carcinoma, and hepatocellular carcinoma. PLK1 LNP is designed to inhibit PLK1 expression, preventing the tumor cell from completing cell division, ultimately resulting in cell cycle arrest and death of the cancer cell.

7. CONCLUSIONS

We are pleased to see that recent advances in lipid gene delivery systems have significantly improved the efficacy and the level of expression of targeted genes, the major barriers limiting the nonviral delivery method. The improved structure–activity design has increased the potential of LNPs in gene therapy in tumors as well as other disorders and diseases. However, the use of LNPs in a clinical setting may only be realistic when there is a better understanding of certain mechanisms and techniques such as: (1) the interaction of lipid vectors and gene expression, (2) the quantitation association between the vectors and gene expression level in vivo, and (3) the safety and immunogenicity profile of vectors. By overcoming these barriers, we will be closer to efficient delivery of genes by LNPs for clinical use.

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CHAPTER THREE

Nanotechnology for *In vivo* Targeted siRNA Delivery

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Contents

Abstract

Small interfering RNAs (siRNAs) can specifically inhibit gene expression. As a result, they have tremendous scientific and clinical potential. However, the use of these molecules in patients and animal models has been limited by challenges with delivery. Intracellular RNA delivery is difficult; it requires a system that protects the siRNA from degradative nucleases in the bloodstream, minimizes clearance by the reticuloendothelial

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system, maximizes delivery to the target tissue, and promotes entry into, and out of, an endocytic vesicle. Despite these barriers, recent data suggest that RNA may be targeted to cells of interest *in vivo*. Herein we outline strategies for targeted siRNA delivery, and describe how these strategies may be improved.

1. RNA POTENTLY MODIFIES GENE EXPRESSION

Once primarily viewed as an intermediary between DNA and protein, RNA is now known to actively regulate gene expression by interacting with DNA, other RNAs, and proteins (Rinn & Chang, 2012; Sabin, Delás, & Hannon, 2013; Ulitsky & Bartel, 2013). Because many of these regulatory functions are dictated by sequence-specific interactions between the RNA sequence and its target, RNAs can precisely modify gene expression and downstream cellular behavior. One well-known example of RNA-mediated gene regulation is RNA interference (RNAi), an endogenous mechanism that reduces protein expression by inhibiting translation of mRNA (Bumcrot, Manoharan, Koteliansky, & Sah, 2006). RNAi is induced by short interfering RNAs (siRNAs) and microRNAs (miRNAs). These small RNAs, which can be introduced into the cytoplasm endogenously by transcription or exogenously through transfection, discourage translation by guiding a protein complex called RISC (RNA-induced silencing complex) to a complementary sequence on the target mRNA (Rana, 2007). While the RNAi pathway has been studied closely for over 10 years, more recent evidence suggests that RNA–RNA interactions can regulate genes through non-RNAi mechanisms. For example, circular RNAs (circRNAs) can sequester miRNAs by binding to them in the cytoplasm (Jeck & Sharpless, 2014).

RNAs can also interact directly with DNA and protein; as a result, RNA-mediated gene regulation does not require RNA–RNA interactions (Sabin et al., 2013). Long noncoding RNAs (lncRNAs) can affect genomic stability by concurrently interacting with DNA and protein complexes that modify the epigenetic state of the cell. For example, an lncRNA named Tsix binds PRC2, a protein complex that modifies histones; another domain of the lncRNA binds to the target DNA, thereby "silencing" DNA expression by recruiting PRC2 to modify chromatin (Sabin et al., 2013). Similarly, RNAs derived from bacterial clustered regularly interspaced palindromic repeats (CRISPRs) can bind to Cas9, a nuclease that induces a double stranded cut in DNA (Sander & Joung, 2014). Once bound to Cas9, the RNA guides the nuclease to a complementary DNA sequence. The result is targeted genomic modification mediated by the DNA–RNA–protein

complex. More simply, RNAs can simultaneously bind two separate proteins and bring them together to activate downstream signaling. These, and other mechanisms reviewed elsewhere, provide strong evidence that RNAs play a fundamental role in cellular function (Rinn & Chang, 2012; Sabin et al., 2013; Ulitsky & Bartel, 2013).

As biologists continue to uncover RNAs that promote health and disease, the number of clinical applications requiring therapeutic RNA delivery will expand. However, to date, effective therapeutic RNA delivery has been limited to siRNAs targeted to hepatocytes of the liver (Kanasty, Dorkin, Vegas, & Anderson, 2013). Therapeutic siRNA delivery has reduced pathological protein in patients with liver diseases including TTR-amyloidosis and familial hypercholesterolemia (Coelho et al., 2013; Fitzgerald et al., 2014). One study showed that nanoparticle-mediated delivery of siRNA targeting TTR reduced serum TTR in humans by nearly 90% after a systemic injection (Coelho et al., 2013). A related formulation reduced low density lipoprotein (LDL) by 57% for one individual in the trial after silencing PCSK9, a gene involved in lipid transport (Fitzgerald et al., 2014). Additional clinical trials that use the same delivery vehicles are planned for other liver diseases, since the biophysical characteristics of the nanoparticles used in these studies do not change with the siRNA sequence. This effect is also illustrated by broad application of the liver-targeting nanoparticle C12-200, which is currently being evaluated for clinical use (Love et al., 2010).

While these nanoparticles convincingly demonstrate that siRNA can affect liver disease in mice, nonhuman primates, and humans, significant needs in the RNA delivery field remain unmet. Most notably, highly efficient delivery to cells outside the liver, and the delivery of longer RNAs to any tissue has remained challenging (Dahlman et al., 2014; Kanasty et al., 2013). Highly efficient *in vivo* delivery requires the material to perform several difficult functions. Without eliciting an unwanted immune response, the material must locate and transfect the target cell in a highly complex and heterogeneous microenvironment (Whitehead, Dahlman, Langer, & Anderson, 2011; Whitehead, Langer, & Anderson, 2009). This requires that the material maximizes interactions with the cell of interest while minimizing similar interactions with nontarget cells and the reticuloendothelial system. A substantial amount of material is typically lost through these unwanted interactions, most notably those interactions with the kidney, liver, and immune system. If the RNA avoids these tissues and reaches the cell of interest, it must get both into and out of an endosome. Even this endocytotic process is inefficient; only 1–2% of siRNA endocytosed by

hepatocytes *in vivo* eventually reached the cytoplasm (Gilleron et al., 2013). The rest of the material was degraded or recycled out of the cell.

The potential for targeted drug delivery vehicles to address important clinical problems has inspired many labs to design nanomaterials for targeted siRNA delivery. For the remainder of this publication, we define targeted delivery systems as those that preferentially transfect certain cells after administration *in vivo*. Delivery can be achieved by active mechanisms (e.g., targeting ligands) or passive mechanisms (e.g., modifying biophysical nanoparticle characteristics like size and charge) (Figure 3.1). As described below, specific strategies within these subsets, each with their own advantages and disadvantages, can be applied to improve siRNA delivery *in vivo*.

2. TARGETING STRATEGIES

2.1 Passive Targeting

As soon as a nanoparticle is injected *in vivo*, it interacts with its environment. If a particle is injected intravenously, the system is initially exposed to blood and endothelial cells that line the vasculature. By contrast, subcutaneous injection exposes the material to the local microenvironment, lymphatic system, and capillary beds near the injection site. These immediate local interactions, and those experienced by the particle as it is transported around the body, can influence where the material is delivered, how well it is delivered, and whether the system induces an unwanted immune response. Put more directly, there is increasing evidence that interactions between particles and the natural physiology of the body can have a substantial effect on the pharmacokinetic profile of the delivery system (Akinc et al., 2010; Monopoli, Aberg, Salvati, & Dawson, 2012; Tenzer et al., 2013).

Enabling natural physiology to passively target siRNA *in vivo* is a promising therapeutic strategy for two reasons. First, passive targeting systems do not contain extraneous active targeting ligands like antibodies, aptamers, or small molecules. This may simplify the synthesis, formulation, and characterization of the delivery system, and thereby reduce batch-to-batch variability. Second, there are many well-characterized differences in physiology that can be exploited for RNA delivery. One such example is the differential structure and function of endothelial cells that line blood vessels throughout the body (Aird, 2006, 2007). Endothelial cells were once considered passive conduits for oxygen and nutrients, but are now known to actively modify metabolism, the immune response, endocytosis, and inflammation by secreting factors and expressing cell-surface receptors (Hagberg

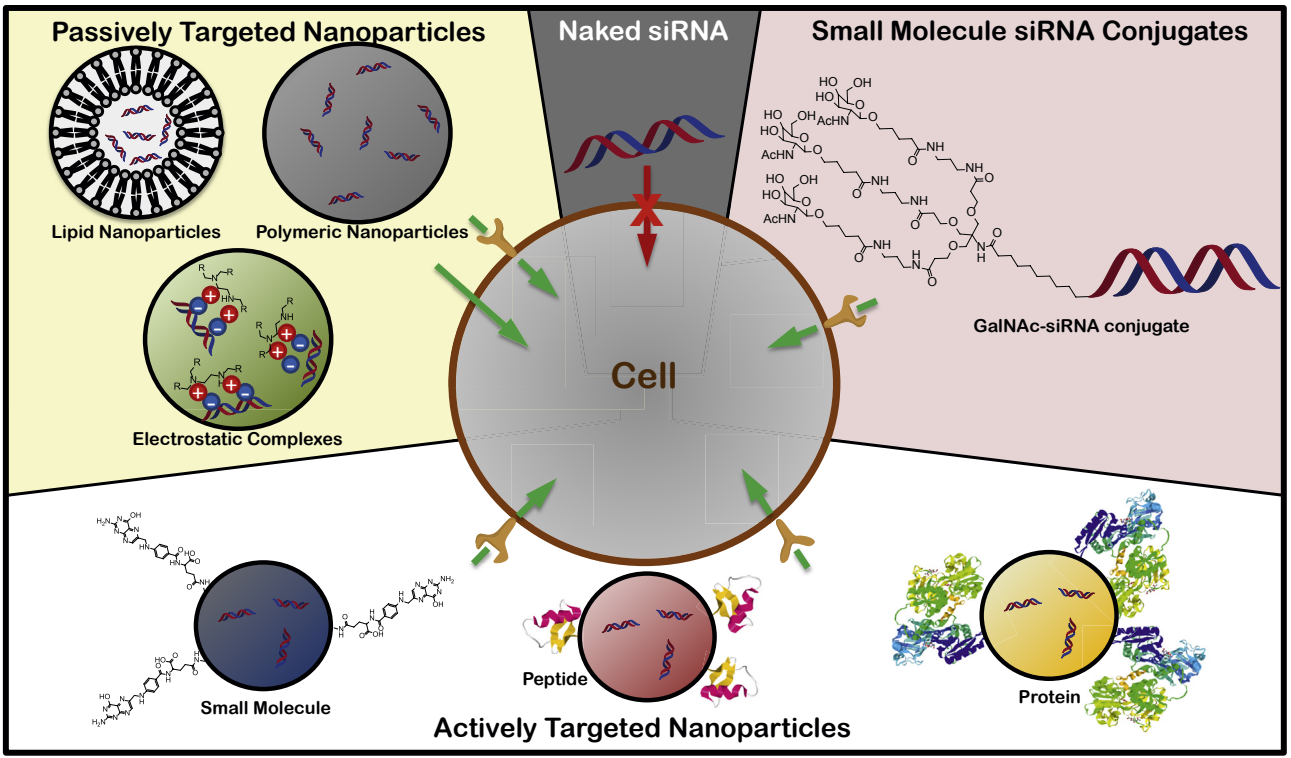

Figure 3.1 ***Strategies to improve siRNA delivery.*** siRNAs, which are large, hydrophilic, and anionic, cannot easily cross the cell membrane by themselves. siRNA delivery can be improved with nanoparticles or conjugates that actively target ligands on the outside of the cell, or by nanoparticles that use natural interactions with the body (e.g., serum proteins) to passively target the cell of interest. (See the color plate.)

et al., 2012, 2010; Pober & Sessa, 2007). In addition to playing a critical role in health and disease, endothelial cells are functionally heterogeneous. The structure, function, and gene expression of these cells vary across different tissues, and within a given tissue (Aird, 2006, 2007). These differences can promote delivery to specific tissues; for instance, delivery to hepatocytes is enhanced by regions of endothelial cells which contain gaps, while delivery to neurons and glial cells in the brain is limited by the tight and continuous barrier of endothelial cells lining the blood–brain barrier. In this same way, natural routes of clearance that increase blood flow to the liver can promote delivery to hepatocytes. The same mechanisms designed to remove and concentrate toxins from the blood can be exploited to concentrate nanoparticles in cells of interest (Akinc et al., 2010).

2.2 Active Targeting

Active targeting systems utilize ligands like proteins or small molecules to bind specific receptors on a target cell surface. The binding can either stabilize the particle on the outside of the cell or trigger receptor-mediated endocytosis and internalization. While many different targeting ligands can be used for targeted siRNA delivery, most scientists have utilized three types of ligands: small molecules, peptides, and proteins (Figure 3.1). Small molecule targeting ligands are molecules with a distinct chemical structure and a molecular weight generally less than 1 kDa. These compounds can mimic natural biomolecules and are synthesized by traditional organic chemistry techniques. Peptide and protein targeting ligands are made of amino acids; peptides consist of less than approximately 50 amino acids while proteins are composed of many more, up to tens of thousands of amino acids. Peptide- and protein-mediated targeting requires precise three-dimensional folding that results from secondary and tertiary protein structures.

Active targeting requires the use of a molecule that binds to a cognate receptor on a target cell. Targeting molecules can be attached to the siRNA directly, however, the synthesis of these siRNA conjugates is challenging. Effective synthesis of siRNA conjugates requires chemical synthesis schemes that meet three criteria. First, reaction conditions that degrade or denature the siRNA or ligand must be avoided. Second, conjugating the siRNA and targeting ligand together cannot reduce the efficacy of either component: the siRNA must still be incorporated into RISC and the ligand must still have specificity for its target receptor. Third, the reaction should generate the highest yield possible so that expensive and inefficient purification is minimized.

One limiting factor in the synthesis of siRNA conjugates is the stability of the siRNA in different chemical reactions. The siRNA must not be denatured during the reaction, since siRNA must remain as a duplex to be properly loaded into RISC and subsequently silence genes (Fire et al., 1998). To maintain RNA integrity, siRNA conjugation reactions should be run in conditions that avoid high temperatures, harsh solvents, and high concentrations of reactive intermediates. One method to avoid denaturing the double stranded RNA during synthesis is to perform conjugation chemistry on the sense strand and then duplex with the antisense strand later. Sense strand modifications have been made to both the 3′ and 5′ end of the siRNA. However, further work is required to understand whether the location of the targeting ligand on the sense strand affects RISC loading and mRNA silencing. Conjugations to the antisense strand should be avoided, since the antisense strand should not have steric hindrances which prevent hybridization with the target mRNA in the RNAi pathway. It is also very important to chemically modify the RNA nucleotides and phosphodiester bonds, as unmodified siRNAs can both induce an immune response and be easily degraded by endogenous ribonucleases (Whitehead et al., 2011). It is common to replace the 2′-hydoxy group on some riboses in the sequence with a 2′-*O*-methyl group or 2′-fluoro and/or to replace one or several phosphodiester bonds with phosphorothioate bonds, although many more modifications have been reported with varying degrees of success (Deleavey & Damha, 2012). These internal modifications are crucial for *in vivo* experiments as they can dramatically both decrease immunogenicity and increase serum stability of the siRNA.

Selecting the right solvent to ligate small molecules to the siRNA can be a particularly important decision. siRNA is soluble in aqueous conditions and precipitates in solutions with too much organic solvent. At the same time, many small molecules commonly linked to siRNA, like cholesterol and folate, are hydrophobic, and therefore require an organic solvent to solubilize. Researchers have overcome this problem using two strategies: first, small molecules and siRNA have been solubilized and reacted in a mixture of water and organic solvent like dimethylsulfoxide or acetonitrile. Second, researchers have attached small molecules during the oligonucleotide synthesis process. For example, cholesterol was conjugated to siRNA by initiating the siRNA synthesis on a controlled-pore glass solid support carrying a cholesterol-aminocaproic acid-pyrrolidine linker; this linker placed a cholesterol on the 3′ end of the sense strand (Soutschek et al., 2004). However, these techniques are limited by the fact that only certain small molecules

are soluble in solvents with aqueous and organic components, many labs do not have access to oligonucleotide synthesis machines, and the solid support method requires optimization for each desired targeting ligand. Reaction conditions that do not affect double stranded siRNA still might denature small molecules, proteins, or oligonucleotides. For example, solvents with aqueous and organic components may differentially attract hydrophobic and hydrophilic regions of a protein, resulting in protein denaturing and loss of function. It is also critical that any modifications to the targeting ligand do not change its ability to bind its receptor. As a result, the active site of the ligand should be identified and conjugations should be performed as far away from this area as possible. Because conjugation reactions change for each targeting ligand, reactions that universally promote conjugation remain an important unmet need.

Despite the strict criteria associated with these reactions, several schemes that successfully conjugate biomolecules have been described (Hermanson, 1996) (Figure 3.2). Many of these reactions utilize common biological functional groups, including amines, carboxylic acids, and thiols. Importantly, siRNAs with these functional groups on the 3′ or 5′ of the sense strand can be purchased from commercial vendors. A bifunctional crosslinker is then used to connect the functional group on the RNA to a different functional group on the targeting ligand. In one example, primary amines are reacted with N-hydroxysuccinimide (NHS) esters to form an amide bond that is stable in physiological pH for several hours. The NHS esters can be generated from carboxylic acids using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry. Another reaction that is commonly used takes place between thiols and maleimide groups; the reaction forms thioether bonds that are stable in physiological pH. Conjugates can also be formed using the highly efficient reaction that takes place between streptavidin and biotin conjugation. This reaction may not be appropriate for smaller targeting ligands, however, because the size of the 53-kDa streptavidin protein can sterically interfere with the targeting ligand. These bioconjugation techniques have been complemented by new approaches that rely on highly efficient and mild "click chemistry." One type of promising click reaction used for siRNA small molecule conjugation links an azide group with an alkyne using a copper catalyst (Yamada et al., 2011). This reaction generates a stable and biologically inert triazole linkage, and can be even performed without the need for a toxic copper catalyst if the alkyne is replaced with a strained cyclooctyne group (Chang et al., 2010). The reaction is rapid and robust, bioorthogonal, and can take place in an aqueous solvent at room temperature.

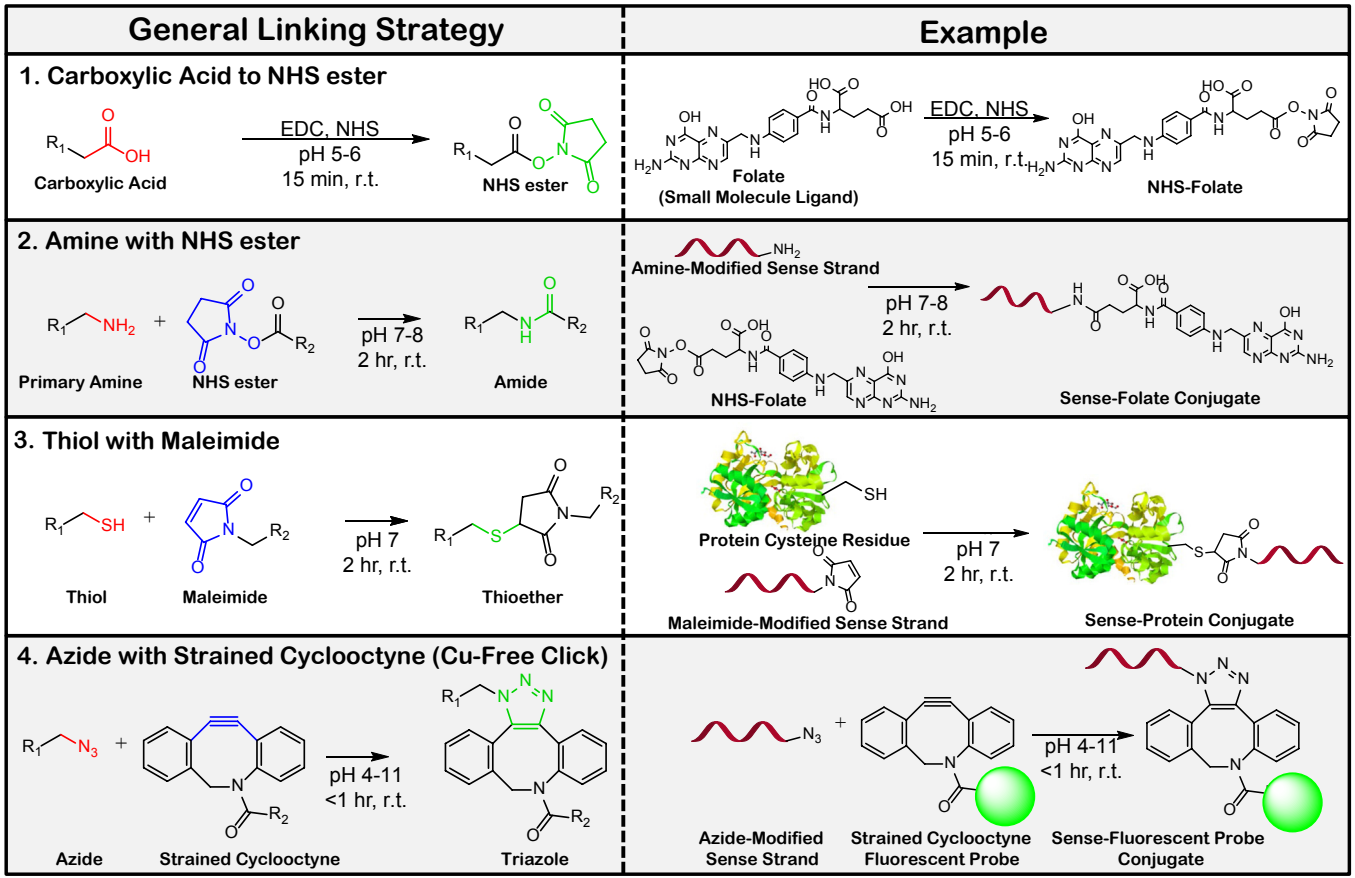

Figure 3.2 ***Chemical reactions to synthesize active siRNA conjugates.*** Conjugation reactions must not denature the targeting ligand, affect siRNA stability, or prevent siRNA loading into RISC. Notably, many synthetic schemes in use today require modification for every new targeting ligand. (See the color plate.)

3. TARGETING THE LIVER

3.1 Liver Physiology Promotes Targeting

Dysfunction of the liver can negatively affect metabolism, glycogen storage, hormone secretion, and serum lipid concentrations (Nolan, Damm, & Prentki, 2011; Rader, Cohen, & Hobbs, 2003). As result, this tissue contributes to a myriad of common diseases that may be amenable to genetic therapies, including cancer, diabetes, clotting disorders, and heart disease. To date, the most clinically advanced siRNA therapies have targeted aberrant gene expression in hepatocytes (Kanasty et al., 2013). The relative ease with which hepatocytes have been targeted can be partially attributed to distinct physiological characteristics that promote hepatocyte delivery. The liver is perfused with the hepatic portal vein, which directs blood gastrointestinal tract to the liver. As a result, delivery systems circulating in the blood have excellent access to the liver. Circulating drugs that flow by the liver can extravasate out of the bloodstream into surrounding liver tissue through nanoscale holes (fenestrae) in sinusoidal endothelial cells (Kanasty, Whitehead, Vegas, & Anderson, 2012). The average diameter of these fenestrae, roughly 100–150 nm depending on the animal species, make fenestrated endothelial cells ideal as a way for nanoscale drug delivery systems designed to reach hepatocytes (Wisse, Jacobs, Topal, Frederik, & De Geest, 2008). Finally, liver delivery mediated by lipid nanoparticles (LNPs) and other hydrophobic systems may be enhanced by the natural mechanisms the liver uses to remove circulating lipids from the bloodstream (Akinc et al., 2010).

3.2 Passive Liver Targeting

The most advanced clinical siRNA-delivery systems utilize LNPs which passively target hepatocytes. One disease that has been treated with LNP formulations is familial hypercholesterolemia, an autosomal dominant genetic disorder characterized by elevated LDL cholesterol. This disease, which greatly increases the risk for cardiovascular disease and sudden death, is driven by overactive PCSK9, a gene whose protein binds to and degrades LDL receptors. A Phase I clinical trial studied the tolerability and efficacy of an LNP developed by Alnylam Pharmaceuticals that was formulated with siRNA targeting the PCSK9 gene (ClinicalTrials.gov, 2011; Fitzgerald et al., 2014). This formulation, termed ALN-PCS02, was tolerated at all tested doses. Moreover, at the highest dose (0.4 mg/kg siRNA) (Fitzgerald et al., 2014) an average of 70% reduction in PCSK9 protein and 40% reduction

in LDL cholesterol from baseline was reported. Using another LNP formulation, Alnylam made nanoparticles targeting transthyretin (TTR), a gene whose gain of function mutations cause TTR-amyloidosis, a debilitating and fatal genetic disease characterized by extracellular deposits of insoluble, misfolded proteins. This formulation was well tolerated in Phase I clinical trials (ClinicalTrials.gov, 2012a, 2010), with no drug related serious adverse events reported. The formulation, called ALN-TTR02, was also effective, reducing TTR levels by over 80% with doses between 0.15 and 0.3 mg/kg in humans (Coelho et al., 2013). Robust and durable protein reduction was also observed with this LNP: TTR serum protein decreased between 57% and 67%, 28 days after treatment. ALN-TTR02 has completed Phase II clinical trials (ClinicalTrials.gov, 2012b) and is currently enrolling patients for a Phase III clinical trial (ClinicalTrials.gov, 2013a).

Incredibly, *in vivo* LNP efficiency with hepatocytes has increased by more than 10,000× within the last 10 years, as shown in Figure 3.3. This rapid improvement has been catalyzed by high-throughput screening and rational design techniques that generate lipids and lipid-like materials that promote delivery (Figures 3.4 and 3.5). These LNPs are typically formulated with three components: (1) cationic or ionizable lipids containing a hydrophilic amine group, a hydrophobic carbon tail, and a chemical linker that binds them, (2) lipid-anchored polyethylene glycol (PEG), which "shields" the LNP against nonspecific uptake by macrophages, increases the circulation time *in vivo* and reduces particle aggregation, and (3) cholesterol or other sterol-like molecules,

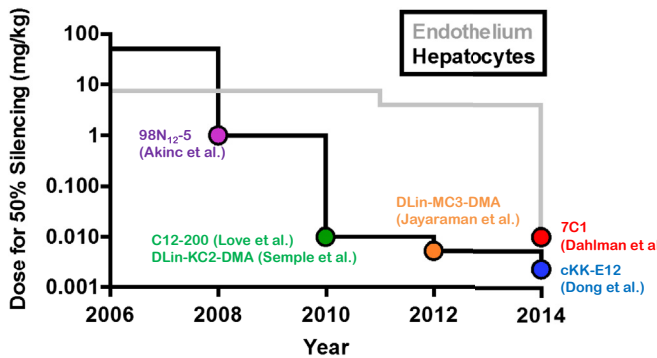

Figure 3.3 ***The efficacy of hepatocyte-targeting siRNA vehicles has improved dramatically over time.*** The dose required to reduce target gene expression in hepatocytes by 50% *in vivo* has decreased more than 10,000× since 2006. Recently, the efficacy of endothelium-targeting siRNA vehicles has been improved by approximately 1,000× with 7C1 nanoparticles (Dahlman et al., 2014). siRNA delivery to other cell types has remained challenging.

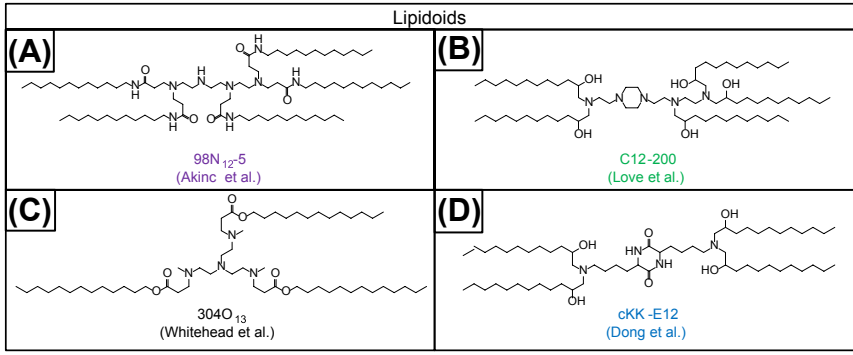

Figure 3.4 ***Multitailed, lipid-like molecules (lipidoids) have successfully delivered siRNA to hepatocytes.*** (A) First-generation lipidoid $N98_{12}$-5 (Akinc et al., 2008), (B) Second-generation lipidoid C12-200 (Love et al., 2010), (C) Biodegradable lipidoid $304O_{13}$ (Whitehead et al., 2014), and (D) and lipopeptide-inspired lipidoid cKK-E12 (Dong et al., 2014).

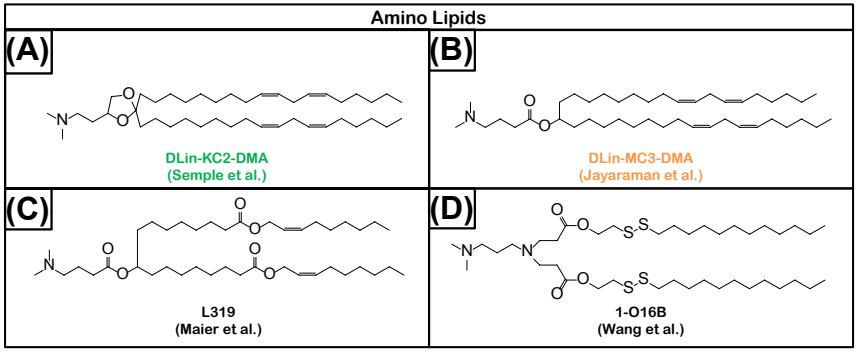

Figure 3.5 ***Two-tailed amino lipids have also successfully delivered siRNA to hepatocytes.*** (A) First-generation amino lipid DLin-KC2-DMA (Semple et al., 2010), (B) Second-generation amino lipid DLin-MC3-DMA (Jayaraman et al., 2012), (C) Biodegradable amino lipid L319 (Maier et al., 2013), and (D) Bioreducible amino lipid 1-O16B (Wang et al., 2014).

which increase LNP stability (Allen & Cullis, 2013; Kanasty et al., 2013). These formulation parameters affect physical properties of the LNP, including size, surface charge, and siRNA loading. Moreover, a growing body of work suggests that the ratio of lipid: PEG: cholesterol: siRNA needs to be tuned to optimize RNA delivery (Akinc et al., 2009).

3.2.1 Lipidoid LNPs

Chemically modifying the components of the LNP can drastically influence biodistribution, efficacy, and tolerability *in vivo*. To investigate the relationship

between lipid structure and *in vivo* function, combinatorial chemistry techniques that permit the rapid synthesis of many multitailed, lipid-like structures (termed "lipidoids") have been developed (Akinc et al., 2008; Dong et al., 2014; Love et al., 2010). Once synthesized, these material libraries are often tested in vitro before the best performing LNPs are selected for further testing *in vivo*. Although in vitro conditions do not recapitulate the complicated *in vivo* environment (Whitehead et al., 2012), this approach has assisted in the discovery of first, second, and third generation hepatocyte-targeting LNPs that reduce target mRNA expression at doses as low as 1.0, 0.01, and 0.001 mg/kg, respectively (Figure 3.3). Increased efficiency reduces the amount of LNP required for delivery, enables more durable gene silencing, and lessens the likelihood of an unwanted immune response. Over the past several years, the generation of lipidoid libraries has resulted in several lead lipidoid compounds (Figure 3.4) discussed in detail below.

The first lead lipidoid, termed $98N_{12}$-5 (Figure 3.4(A)), was selected from a library of over 1200 lipidoids (Akinc et al., 2008). The library was synthesized by conjugating small amines to lipids terminated with acrylates or acrylamides via the Michael addition reaction. This reaction was selected because it was robust, required one step, and did not require complicated or expensive purification. $98N_{12}$-5 LNPs were formed by mixing $98N_{12}$-5 with PEG, cholesterol, and siRNA. This LNP was formulated with siRNA targeting Factor VII, a blood clotting factor produced specifically by hepatocytes and easily measured in serum. $98N_{12}$-5 mediated delivery reduced Factor VII serum concentration by over 90% in mice after two daily IV 2.5 mg/kg doses; similarly, when formulated with siRNA targeting apolipoprotein B (ApoB), $98N_{12}$-5 reduced ApoB mRNA expression by 85% in nonhuman primates after a single IV 6.25 mg/kg injection. In addition to identifying a lead candidate, this high-throughput screening technique revealed structural features that were shared by top-performing compounds in the study. Effective compounds had amide bonds linking the lipid tails and amines, more than two alkyl tails with 8–12 carbons, and at least one secondary amine.

The structure–function relationships from the first lipid library informed the synthesis of second-generation lipids that dramatically improved delivery to hepatocytes *in vivo*. Once again, simple and robust chemistry allowed for the rapid generation of a structurally diverse library that was screened in vitro (Love et al., 2010). A lead lipidoid termed C12-200 (Figure 3.4(B)) had an IC_{50} of approximately 0.01 mg/kg and reduced Factor VII serum in mice by nearly 100% after a 0.1 mg/kg injection. C12-200 was also effective

in nonhuman primates, reducing TTR serum protein by 90% after a single injection of 0.3 mg/kg. C12-200 further enabled highly durable Factor VII protein silencing in mice, and facilitated the first reported multigene *in vivo* knockdown after it was concurrently formulated with five different siRNAs, each targeting a different hepatocyte-specific gene (Love et al., 2010).

To study whether biodegradable linkages could further improve *in vivo* tolerability, another lipidoid library was then synthesized using alkyl acrylate tails, which contain ester groups capable of hydrolysis by liver enzymes, to make biodegradable lipidoids (Whitehead et al., 2014). As predicted, exposing the new biodegradable lipidoid to hydrolytic conditions resulted in degradation into the predicted alkyl-alcohol products. siRNA-loaded LNPs made with the lead degradable lipidoid, $304O_{13}$ (Figure 3.4(C)), had an IC_{50} of 0.01 mg/kg, which is equivalent in potency to C12-200. To determine if enhanced degradability translated to better tolerance *in vivo*, the authors dosed $304O_{13}$ and C12-200 LNPs at levels 100-fold their IC_{50}. $304O_{13}$ LNPs produced less immune response (lower cytokine levels) than C12-200 at this high dose, and liver histology showed necrosis and inflammation in mice dosed C12-200 at 1 mg/kg but none for $304O_{13}$ dosed at 5 mg/kg. Furthermore, the authors identified "efficacy criteria" for this class of lipidoids: potent lipidoids had a tertiary amine with at least three tails of 13 carbons, but the most important LNP parameter was having a surface pKa of at least 5.5.

Inspired by the fact that the liver naturally takes up lipoproteins from the blood, we recently synthesized a library of lipopeptide-based materials (Dong et al., 2014). These biomimetic compounds were synthesized by reacting lipids with different functional groups to the free amine on small peptides. After screening the library in vitro, we identified a lead candidate, termed cKK-E12 (Figure 3.4(D)), that reduces FVII serum protein by 50% after a single injection of 0.002 mg/kg cKK-E12 also reduced TTR serum concentration in nonhuman primates by 95% after a single injection of 0.3 mg/kg cKK-E12 selectivity toward hepatocytes and tolerability was also measured *in vivo;* at low doses it did not transfect endothelial cells or immune cells *in vivo*, and resulted in no apparent toxicity. Lipopeptide structure–function relationships were again studied. The most effective compounds commonly had a lysine-based ring structure and a lipid tail between 12 and 14 carbons bound by an epoxide- or aldehyde-terminated lipid.

3.2.2 Amino Lipid LNPs

Whereas multitailed lipidoids have been generated primarily via high-throughput combinatorial libraries, a second class of lipids, two-tailed

ionizable "amino lipids" (Figure 3.5), have been generated with a rational-design approach. To synthesize the compound DLin-KC2-DMA (Figure 3.5(A)), each section of the lipid (polar head and linker group) was individually optimized by systematically varying parameters like carbon chain length and hydrophilicity (Semple et al., 2010). A previous study had already optimized the tail region of the amino lipid, finding that 18-carbon tails with two-double bonds ("DLin") had the best gene silencing in vitro (Heyes, Palmer, Bremner, & MacLachlan, 2005). LNPs formulated with DLin-KC2-DMA, cholesterol, PEG, and siRNA reduced Factor VII serum protein by 90% after a single injection of 0.1 mg/kg in mice and TTR serum protein by 80% after a single 1.0 mg/kg injection in nonhuman primates (Semple et al., 2010). The IC_{50} of DLin-KC2-DMA LNPs was 0.01 mg/kg, equivalent to the lipidoid C12-200: interestingly, two different approaches (high-throughput vs rational design) and two different lipid-inspired chemical structures (multitailed lipidoids vs two-tailed amino lipids) resulted in siRNA-delivery compounds with nearly identical potency *in vivo*.

Since the initial report of DLin-KC2-DMA, further work has been performed to improve LNP formulation and characterize the particle. The formulation process used to synthesize DLin-KC2-DMA LNPs has been improved through the use of microfluidic mixing devices, which produces LNPs with more reproducible size, higher siRNA encapsulation efficiency, and larger scale than traditional pipet-mixed particles (Belliveau et al., 2012). Importantly, these LNPs made with microfluidic mixing devices were as potent *in vivo* as particles made with traditional mixing methods. Computational modeling, cryo-TEM, and other techniques used to study the internal structure of these microfluidic-mixed DLin-KC2-DMA LNPs revealed that the nanoparticle structure is made of siRNA complexed to cationic lipid and periodically spaced aqueous regions inside the LNP core (Leung et al., 2012). Since its initial report, DLin-KC2-DMA has also been used to passively deliver siRNA to nonhepatocellular targets. By increasing the size of the LNP from 80 nm diameter to 360 nm diameter (much larger than the 100–150 nm fenestrae in liver endothelium), the authors reduced siRNA delivery to liver and allowed for more selective delivery *in vivo* to antigen-presenting cells like bone marrow macrophages and dendritic cells, which readily endocytose particles less than 10 μm in size (Basha et al., 2011).

Second-generation amino lipid based on DLin-KC2-DMA were then synthesized (Jayaraman et al., 2012). The amine head group and linker section of the amino lipid were further modified to modulate the pKa; 53 new amino lipids with pKa values ranging from 4.17 to 8.12 were generated.

These amino lipids were complexed with siRNA (without PEG, cholesterol, or other excipients) and screened in mice via intravenous injection. The authors found a remarkable correlation between pKa and potency: pKas between 6.2 and 6.5 had the lowest IC_{50} for all amino lipids tested, and efficacy rapidly decreased below 6.2 and above 6.5. This amino lipid pKa criterion corroborates the pKa criterion found with biodegradable lipidoids, which was that pKa must be 5.5 or greater (Whitehead et al., 2014). The lead compound discovered in the *in vivo* screen was called DLin-MC3-DMA (Figure 3.5(B)), which had a pKa of 6.44 and an IC_{50} of 0.005 mg/kg when incorporated into an LNP formulation with helper lipid, cholesterol, and PEG (Jayaraman et al., 2012). It should be noted that the previously mentioned clinical trials led by Alnylam Pharmaceuticals to treat hypercholesterolemia (ClinicalTrials.gov, 2011) and TTR-amyloidosis (ClinicalTrials.gov, 2012b) (Section 3.2) both use an LNP formulation based on DLin-MC3-DMA.

A biodegradable version of DLin-MC3-DMA was then synthesized by replacing one of the double bonds in the hydrocarbon tail with a degradable ester linkage (Maier et al., 2013). It was found that moving this ester bond farther up the tail toward the headgroup decreased *in vivo* efficacy. The lead biodegradable amino lipid in the initial screen was called L319 (Figure 3.5(C)) and had an IC_{50} of less than 0.01 mg/kg when formulated in LNPs, similar to its nonbiodegradable counterpart DLin-MC3-DMA. Through pharmacokinetic studies in mice, it was demonstrated that L319 is degraded through ester cleavage into hydrophilic, water-soluble metabolites which are rapidly cleared from plasma and tissues. Furthermore, toxicology studies in rats showed no significant increase in liver toxicology markers alanine transaminase (ALT) and aspartate transaminase (AST) up to a 10 mg/kg dose of L319 LNPs, which is 1000× higher than the IC_{50}, showing a very large therapeutic window; in comparison, nonbiodegradable control LNPs showed elevated ALT and AST levels above 3 mg/kg. Because enzyme profiles differ across species, nonhuman primates were used to confirm L319 LNP efficacy (70% TTR knockdown at 0.3 mg/kg) and rapid clearance of L319 from plasma (30,000× reduction in 24 h) in a higher-order species.

A separate report employed a different strategy to create degradable amino lipids: both ester bonds and disulfide bonds were incorporated into the hydrocarbon tails (Wang et al., 2014). Whereas esters can be hydrolyzed by enzymes in the body, disulfide bonds can be broken into thiols in the reducing environment of the cytoplasm. A small set of amino lipids with these biodegradable and bioreducible bonds were synthesized, and a lead

candidate named 1-O16B (Figure 3.5(D)) was identified. For the six compounds tested through complexation with siRNA, all six had statistically significant increases in gene knockdown in vitro when a disulfide bond was incorporated into the tails. It was hypothesized that this increased efficacy is caused by the disulfide bond reduction in the cytoplasm, triggering siRNA release from the electrostatic complex as the amino lipid degrades more rapidly. Behavior of 1-O16B and similar bioreducible amino lipids *in vivo* has not yet been reported.

3.2.3 Endocytosis of Lipidoid and Amino Lipid LNPs

LNP-mediated delivery is thought to exploit the natural endocytosis of serum apolipoproteins, however, the precise mechanism governing LNP cellular endocytosis by hepatocytes is poorly understood and likely varies with the chemical nature of a given LNP. Some LNPs use an apolipoprotein-E (ApoE) dependent mechanism for hepatocyte uptake; it is postulated that protein ApoE adsorbs on to the LNP, directs the LNP to hepatocytes, and binds its natural receptor, LDL receptor (LDLR). Once bound to the LDLR receptor, the ApoE and the LNP are concurrently taken into the hepatocyte via receptor-mediated endocytosis. To support this mechanism, LNPs made from DLin-KC2-DMA were injected into wild-type, $ApoE^{-/-}$, and $LDLR^{-/-}$ mice (Akinc et al., 2010). The LNP was highly potent in normal mice, but did not deliver siRNA in $ApoE^{-/-}$ knockout mice or $LDLR^{-/-}$ knockout mice. Moreover, delivery efficiency was rescued when recombinant ApoE was administered to the $ApoE^{-/-}$ mice. This same methodology revealed that cKK-E12 uptake was also ApoE-dependent. Interestingly, LNPs made from the lipidoid $N98_{12}$-5 still transfected $ApoE^{-/-}$ mice, suggesting some other form of internalization. Although not conclusive, these studies suggest that endocytosis may be impacted by LNP surface charge; those with near neutral surface charge at physiological pH may be more likely to undergo an ApoE-dependent endocytosis mechanism.

3.3 Active Liver Targeting

Complementing LNPs which passively target the liver are systems that have been designed to actively target hepatocytes by binding to the asialoglycoprotein receptor (ASGPR). This receptor, which is constitutively and specifically expressed on the surface of hepatocytes, has a carbohydrate recognition domain that binds to the monosaccharide galactose and plays an important role in glycoprotein homeostasis (Meier, Bider, Malashkevich, Spiess, & Burkhard, 2000). Binding between galactose and ASGPR is well

characterized: alcohol groups at the 3- and 4- positions of the galactose bind to ASGPR by interacting with a calcium ion in the ASGPR and forming hydrogen bonds with neighboring amino acids (Weis, 1996). This binding changes the configuration of the receptor and triggers receptor-mediated endocytosis. Because this receptor is expressed primarily on hepatocytes, several labs have developed galactose-analog conjugates for the delivery of siRNAs and other therapeutics (Jain, Kesharwani, Gupta, & Jain, 2012). This interest has grown as recent evidence suggests that these analogs can effectively deliver therapeutics to hepatocytes without significantly transfecting Kupffer cells or other tissues *in vivo* (Spiess, 1990).

Hepatocyte-targeting with galactose and its analogs has improved with our understanding of the mechanisms that govern the interaction between the ligand and its receptor. Early work relied on a cationic polymer polyethyleneimine (PEI) that was conjugated to galactose and complexed with DNA (Kim et al., 2005). This compound improved DNA delivery to hepatocytes *in vivo,* but was limited by the inherent toxicity associated with high molecular weight PEI. To improve selectivity and tolerability, groups utilized the ligand *N*-acetylgalactosamine (GalNAc), a simple derivative of galactose with an acetylamino group replacing the hydroxyl at the 2-position of the sugar, that binds to the ASGPR receptor with higher selectivity than unmodified galactose (Drickamer, 1996). This engineering approach was used to study whether additional chemical modifications made to the 2- and 6- positions of GalNAc increased the binding affinity to ASGPR. Indeed, when trifluoroacetyl modifications were made to the 2- position GalNAc, the binding affinity for ASGPR increased by more than 50-fold (dissociation constant K_d = 0.7 vs 40 μM, respectively) (Mamidyala et al., 2012). Because this binding study was performed without RNA, it will be important to understand whether increased affinity is still observed with conjugated siRNA.

The binding of GalNAc to ASGPR has also been improved by increasing the valency of the GalNAc ligand. It has been shown that binding affinity increases when clusters of glycoside receptors are simultaneously bound with an optimal spacing of at least 15 Å between sugar residues (Rensen et al., 2001). To take advantage of this clustering effect, triantennary GalNAc was synthesized; its ASGPR dissociation constant was 2 nM, 2000-fold lower that than the single GalNAc system (Rensen et al., 2001). Triantennary GalNAc conjugated directly to the 3′ end of the sense strand of siRNA has been used by Alnylam Pharmaceutics in clinical trials. The most clinically advanced triantennary GalNAc-siRNA conjugate is ALN-TTRsc, a

subcutaneously administered therapeutic for treatment of TTR-mediated amyloidosis (Alnylam, 2013). No significant adverse effects were observed in Phase I clinical trials, and TTR serum protein was reduced in patients treated with 2.5 mg/kg dose (Alnylam, 2013; Butler et al., 2014). Increasing the dose to 10 mg/kg resulted in more potent TTR protein reduction; up to 94% protein reduction was measured after a single dose. Although these siRNA doses are much higher than Alnylam's lipid nanoparticle TTR formulation (ALN-TTR02), the direct GalNAc-siRNA conjugates were well tolerated at doses well above those needed for potent silencing. Moreover, these targeted conjugates did not require PEG or cholesterol, and were administered subcutaneously instead of intravenously. This makes patients more likely to tolerate the injection, and in turn, increases the number of clinical indications to which these systems can be applied. Alnylam is currently utilizing the GalNAc conjugates to reduce PCSK9 and antithrombin (AT) to treat familial hypercholesterolemia and hemophilia, respectively.

GalNAc targeting ligands have also been used by the Dynamic Polyconjugate (DPC) system (Rozema et al., 2007; Wong et al., 2012; Wooddell et al., 2013). First-generation DPCs (Rozema et al., 2007) used an amphipathic polymer made of butyl and amino vinyl ethers (PBAVE) as a reactive backbone to which siRNA, the GalNAc targeting ligand, PEG were attached via acid-labile linkages. While the exact mechanism of action remains unclear, it is hypothesized that these linkages break in the acidic environment of the endosome, allowing the newly "unmasked" amine groups on the PBAVE to help destabilize the membrane and facilitate endosomal escape. First-generation DPCs administered intravenously to mice at a 2.5 mg/kg siRNA dose reduced ApoB by 80–90%. This potent silencing was accompanied by a phenotypic reduction in serum cholesterol (Rozema et al., 2007). Slight but nonsignificant increases of liver enzymes and cytokines were observed during the study; however, the authors concluded DPC was well tolerated. Based on the hypothesis that a biodegradable polymer would decrease toxicity, Merck synthesized a bioreducible variant of the DPC system by incorporating disulfide bonds in the polymer (Parmar et al., 2013). This compound reduced ApoB mRNA expression by 80% in mice after a 3 mg/kg injection.

Recently, it was reported that the PBAVE polymer need not be covalently bound to the siRNA for efficient hepatocyte gene silencing (Wong et al., 2012). When authors co-injected PBAVE and a cholesterol-siRNA conjugate, they found that both systems co-localized to mouse hepatocytes and silenced genes, even when the polymer and siRNA were injected 2 h

apart. Injecting the endosomolytic agent and cholesterol-siRNA improved the efficiency by 500-fold compared to cholesterol-siRNA alone and greatly simplified the formulation process. A second-generation delivery system utilizing a GalNAc conjugated to a small peptide called melittin, a small biodegradable peptide component of bee venom which is thought to disrupt the endosome and enhance endosomal escape. This system delivered siRNA that reduced chronic Hepatitis B virus (HBV) infection in mice (Wooddell et al., 2013). These results demonstrated that melittin promoted delivery without generating anti-melittin antibodies. The successful mouse and nonhuman primate results of this study led to a Phase I clinical study studying the safety and tolerability of a coinjection of GalNAc/masked-melittin conjugates with siRNA-cholesterol conjugates (called ARC-520) to treat chronic hepatitis B infection in humans (ClinicalTrials.gov, 2013b). In March 2014, a Phase IIa clinical trial of ARC-520 was started in patients with chronic HBV (ClinicalTrials.gov, 2014).

GalNAc ligands have also been used to actively target nanoparticles to hepatocytes. Triantennary GalNAc was incorporated into DLin-KC2-DMA nanoparticles and injected *in vivo* (Akinc et al., 2010). This conjugation significantly improved siRNA delivery compared to unmodified DLin-KC2-DMA nanoparticles in ApoE$^{-/-}$ mice, which could not use the endogenous ApoE-dependent endocytosis mechanism and thus forced ASGPR-mediated endocytosis.

4. TARGETING PRIMARY TUMORS AND METASTASIS

4.1 Tumor Physiology can Promote or Inhibit Delivery

The term cancer encompasses hundreds of complicated diseases with distinct presentations, available treatments, and prognoses. At the most basic level, cancers are characterized by aberrant and uncontrolled cell growth. As mutated cells continue to grow more rapidly and die less often than normal cells, the physiology of the resulting primary tumor becomes unstable and heterogeneous. The complicated genetic and phenotypic landscape of primary tumors (and their metastases) often creates an environment that can either promote or prevent the delivery of nanotherapeutics. Tumor vasculature, for instance, can be discontinuous, twisted, and leaky (Carmeliet & Jain, 2011). This occurs because the synthesis of normal functional blood vessels requires an intricate cascade of molecular signals that occur in specific order both in space and time (Herbert & Stainier, 2011). Abnormal signaling that takes place in the tumor prevents the normal signaling cascade

from being completed, leading to the rapid generation of dysfunctional, tortuous vessels. While leakiness may enhance the delivery of some drug delivery systems in certain animal models, there is also strong evidence that suggests just the opposite: because the tumor vessels had been leaky for some time and new cells were being generated rapidly in the tumor, hydrostatic pressure builds up in the tumor microenvironment (Jain, 2005). As a result, nanoparticles are not able to exit tumor vessels. Delivery may also be affected by the immunological state of the tumor. Tumor physiology is proinflammatory; as a result, tumors are often filled with immune cells, which can endocytose nanoparticles (Fridman, Pagès, Sautès-Fridman, & Galon, 2012). Finally, the cancer cells and co-opted cells of the tumor microenvironment are often mutated, leading to abnormal cellular function. Cells can undergo mutations that can either promote or inhibit endocytosis and cytoplasmic delivery. Taken together, these factors suggest that the relationship between drug delivery and tumor physiology will remain contentious, and will likely depend on the specific tumor and type and *in vivo* model (Carmeliet & Jain, 2011; Jain, 1994).

Primary tumors can also shed cells into the circulation. A small fraction of these cells can spread to distant organs through a process called metastasis. This process has been difficult to treat therapeutically because surgical resection and localized radiation are often not viable options when the disease has spread. As a result, over 90% of cancer deaths result from metastasis. Metastasis is a relatively complicated, and therefore inefficient, biological process that requires cells to clear a path to the vasculature, enter the circulation, exit the circulation, and proliferate in the secondary site (Chiang & Massagué, 2008; Gray, 2010; Nguyen & Massagué, 2007). Metastasis is initially promoted by primary tumor growth. Rapid growth leads to the formation of dysfunctional and inefficient blood vessels; poor perfusion in the tumor prompts cancer cells to express genes that induce cell motility and anaerobic metabolism (Chiang & Massagué, 2008; Nguyen & Massagué, 2007). In some cases, cancer cells can actively degrade extracellular matrix and epithelial cell–cell junctions in their surrounding microenvironment to clear a path toward nutrient-rich vasculature. Once at the vasculature, the cells can enter the bloodstream by degrading endothelial cell–cell junctions or increasing endothelial cell permeability by releasing molecules like vascular endothelial growth factor. Metastatic cells in the bloodstream can bind circulating platelets and leukocytes to increase survival before they reach their secondary site. At the secondary site, the cells must extravasate out of the circulation, survive in an unfamiliar microenvironment, and

proliferate. Cancer cells exit the circulation in a process akin to the one they use to enter it; they release molecules that induce endothelial cell death and degrade the surrounding extracellular matrix. At that point, the metastatic cells can co-opt natural growth signals in the microenvironment to enhance their own survival and growth. For example, metastatic breast cancer cells embedded in bone marrow express CXCR4 receptors that bind to nearby CXCL12 ligands, leading to metastatic progression (Wang, Loberg, & Taichman, 2006).

Each step of metastatic process occurs in a different part of the body (e.g., primary tumor, bloodstream, secondary site), relies on the activation of different genetic programs, and takes place in patients who are already sick and weakened (Schroeder et al., 2012). As a result, siRNA therapies targeting metastatic disease will require highly advanced delivery systems that can be targeted to different parts of the body safely.

4.2 Active Tumor Targeting

Aberrant gene expression in tumors can result in the overexpression of cell-surface receptors that can be actively targeted. One such receptor-used active-targeted tumor delivery is the folate receptor. This receptor is expressed in low levels on healthy cells, but is overexpressed in epithelial cancers (including those of the ovary, colon, lung, prostate, nose, throat, and brain) and hematopoietic malignancies of myeloid origin (including myelogenous leukemias) (Hilgenbrink & Low, 2005). Binding between the folate receptor and its ligand (folate, also termed folic acid) initiates downstream signaling that promotes cell survival and proliferation. The utility of folate as a ligand is enhanced by its γ-carboxylic acid. This carboxylic acid has been directly conjugated to nanoparticles, siRNA, and even DNA origami structures that were simultaneously bound to siRNA (Lee et al., 2012). In all cases, delivery to cells with folate receptors was greater than delivery to cells without the receptor. For example, one study found that mesoporous silica nanoparticles conjugated with folate delivered drugs more effectively to pancreatic xenograft tumors in mice than nanoparticles than without folate (Lu, Li, Zink, & Tamanoi, 2012). Similarly, siRNA that was directly conjugated to folate via a low molecular weight PEG spacer showed increased RNA delivery compared to nonconjugated siRNA (Dohmen et al., 2012).

In addition to the 441-Da folate molecule, the 78-kDa glycoprotein transferrin can also be used in cancer-targeting RNA therapies. The transferrin receptor, which affects ion transportation and cell growth, is

overexpressed on malignant cells (Tortorella & Karagiannis, 2014). Transferrin was used to target a linear, cationic cyclodextrin polymer formulated into nanoparticles which preferentially deliver siRNA to tumors *in vivo* (Bartlett & Davis, 2008; Davis et al., 2010; Gonzalez, Hwang, & Davis, 1999; Heidel, Yu, et al., 2007). The therapeutic target for the incorporated siRNA is the M2 subunit of ribonucleotide reductase (RRM2), an enzyme whose inhibition reduces cancer cell proliferation both in vitro and *in vivo* for humans and other species (Heidel, Liu, et al., 2007). The transferrin targeting ligand is crucial for potency: at 2.5 mg/kg dosing in mice, RRM2-siRNA-loaded cyclodextrin nanoparticles formulated with the transferrin ligand slowed tumor growth, while identical particles formulated without the transferrin ligand did not (Bartlett & Davis, 2008). A study was performed in non-human primates to determine the tolerability of these cyclodextrin-based nanoparticles with siRNA (Heidel, Yu, et al., 2007). Doses up to 9 mg/kg appeared to be well tolerated with no detectable toxicity, whereas doses at 27 mg/kg produced a mild immune response, an unsurprising result considering the authors used siRNA which contained no chemical modifications to reduce immunogenicity.

The transferrin-targeted, cyclodextrin-based nanoparticles with RRM2-siRNA (called CALAA-01) were used in clinical trials and produced the first direct evidence of RNAi in humans (ClinicalTrials.gov, 2008; Davis et al., 2010). The nanoparticles were injected IV into patients with solid cancers refractory to standard-of-care therapies at mg/kg on days 1, 3, 8, and 10 of a 21-day cycle. Preliminary results from the Phase I clinical trials showed that the treatment reduced RRM2 mRNA and protein levels and induced mRNA cleavage, as measured by 5′-RLM-RACE. Unfortunately, in 2013 this Phase I clinical trial was terminated (ClinicalTrials.gov, 2008), the reasons for which were not reported.

Active siRNA delivery to ovarian cancer cells has been achieved with siRNA conjugated to tumor-targeting peptides. More specifically, the cyclic nonapeptide LyP-1, found through an *in vivo* screen of random peptides displayed on phage surfaces, targeted lymphatic endothelial cells in tumors and by selectively binding to HABP1, a mitochondrial protein overexpressed in ovarian and other tumors (Fogal, Zhang, Krajewski, & Ruoslahti, 2008). Lyp-1 has also been modified to increase functionality: dual peptides with Lyp-1 domains and transportin domains were synthesized to promote tumor penetration and membrane transport, respectively (Ren et al., 2012). The tandem peptide was complexed with siRNA targeting ID4, an oncogene that was found to be essential for proliferation in many ovarian cancers

(Ren et al., 2012). The conjugates were then formulated into nanoplexes and injected in the peritoneal cavity or intravenously with ovarian tumor-bearing mice. The particles penetrated deeply into tumor parenchyma, reduced tumor ID4 mRNA expression by 80%, and significantly reduced tumor growth. These delivery systems also distributed to the liver, spleen, and lung. Although intraperitoneal injection showed better accumulation in the ovarian tumor, there was not a significant difference in ID4 knockdown between the two routes of administration.

5. ENDOTHELIAL CELL TARGETING

Endothelial cells, which line the blood vessels that penetrate nearly every tissue in the body, actively influence blood pressure, inflammation, metabolism, angiogenesis, and microenvironmental regulation. As a result, these cells contribute to more disease than any other tissue in the body, including cardiovascular ischemia, cerebrovascular ischemia (e.g., stroke), primary tumor growth, metastasis, diabetes, and chronic inflammation (Kumar, Abbas, Fausto, & Aster, 2009). Because there are many diseases that would benefit from highly efficient endothelial siRNA delivery, a number of delivery systems have been designed to deliver siRNA to endothelial cells *in vivo* (Kaufmann, Ahrens, & Santel, 2010). LNPs made from a cationic lipid, a helper lipid, and PEG were injected intravenously at a dose of 1.88 mg/kg on four consecutive days. Following this total dose of 7.52 mg/kg siRNA, pulmonary and hepatic endothelial cell mRNA and protein decreased significantly (Santel, Aleku, Keil, Endruschat, Esche, Durieux, et al., 2006; Santel, Aleku, Keil, Endruschat, Esche, Fisch, et al., 2006). Second-generation LNPs were then shown to reduce pulmonary endothelial cell mRNA expression after a total dose of 2.8 mg/kg siRNA (Aleku et al., 2008). While these delivery systems effectively targeted pulmonary endothelial cells *in vivo*, they required doses much higher than those required for potent hepatocyte silencing (0.01 mg/kg), as shown in Figure 3.3.

Inspired by the highly efficient hepatocyte delivery, a library of low molecular lipid-polymer materials was developed for endothelial delivery. Formulations were first screened them for their ability to reduce target mRNA expression in multiple cell lines, including two endothelial cell lines. Lead candidates were screened *in vivo,* leading to selection of a compound termed 7C1, a low molecular weight compound made by conjugating C_{15} epoxide-terminated lipids and extremely low

molecular weight (M_N = 600 Da) branched PEI (Dahlman et al., 2014). 7C1 nanoparticle formulation was optimized to ensure particles were small, stable, and repeatedly silenced target mRNA *in vivo*. For example, seven batches of 7C1 nanoparticles were made with either extrusion or microfluidic mixing, and injected intravenously. Particles made with microfluidic mixing silenced mRNA expression much more consistently than those made with extrusion. The 7C1 formulation was then made with different 7C1: cholesterol: PEG molar ratios; unlike liver-targeting compounds, 7C1 did not require cholesterol to maximize mRNA silencing. The optimized 7C1 formulations potently reduced target mRNA expression in pulmonary, cardiovascular, and renal endothelial cells *in vivo* after injections of 0.017, 0.04, and 0.08 mg/kg, respectively. These compounds did not reduce target mRNA expression in pulmonary immune cells, systemic immune cells, or hepatocytes *in vivo*. The precise mechanism governing the preferential targeting of 7C1 particles to endothelial cells is currently unclear, but may be related to interactions with serum proteins. 7C1 delivery also reduced target mRNA expression for over 21 days following one injection, and to simultaneously deliver siRNAs targeting five different genes concurrently *in vivo*.

The functional effect of 7C1-mediated endothelial siRNA delivery was confirmed in animal models of vascular permeability, emphysema, primary tumor growth, and metastasis (Dahlman et al., 2014). However, in all these cases, a single siRNA was used to elicit a desired phenotype. Because multiple small RNAs can be formulated into a single 7C1 nanoparticle, we investigated whether targeted combination therapy could reduce disease progression and extend survival in a genetically engineered mouse model of nonsmall cell lung cancer (NSCLC) (Xue et al., 2014). A clinically relevant spontaneous tumor model in which lung epithelial cells simultaneously express tumorigenic $Kras^{G12D}$ was investigated. These so-called KP tumors are extremely aggressive, and mimic both human lung cancer progression and response to therapeutics (Meylan et al., 2009). 7C1 was first formulated with a therapeutic miRNA called miR-34a that is downregulated in NSCLC. miR-34a replacement significantly reduced tumor growth without inducing measurable increases in serum cytokine expression. 7C1 nanoparticles were then formulated with siRNA targeting Kras, an oncogene that drives tumor progression and metastasis. siKras therapy significantly slowed tumor growth, and increased tumor apoptosis. The combination therapy with both miR-34a and siKras resulted in tumor regression and significantly extended

survival on its own and when used in concert with cisplatin, a first-line NSCLC therapy (Xue et al., 2014).

6. FUTURE PERSPECTIVES

Delivery systems that have been designed over the past 10 years have significantly improved the likelihood that successful siRNA therapies will be approved for clinical use. Many of the most clinically advanced delivery systems target hepatocytes. As a result, patients with a myriad of liver diseases stand to benefit from RNAi therapies. These advances are particularly exciting given the number of severe genetic liver diseases driven by a small number of genes that significantly diminish the quality of life (e.g., TTR-amyloidosis, hemophilia, and porphyria). Successful long-term inhibition of these genes may effectively cure these diseases. Because the biodistribution, pharmacokinetics, and safety profile of the administered drugs will not vary significantly with siRNA sequence, the same delivery vehicles may be used to treat liver diseases that are driven by a specific collection of genes with RNA combination therapies. Finally, as delivery systems that are administered subcutaneously continue to improve, siRNA therapies may also be used to treat more common diseases like hypercholesterolemia. For instance, inactive forms of ApoC3 or PCSK9 may significantly reduce serum cholesterol concentrations, and resultant cardiovascular disease. Pharmaceutical companies are developing antibody therapies that block the extracellular component of these targets. However, if subcutaneous conjugate delivery systems are shown to be safe and effective, these blockbuster antibodies may need to compete with siRNA therapies that "delete" both the extracellular and intracellular components of the protein target.

As RNA therapies become more commonplace in the clinic, they may be used in concert with traditional drugs. This effect is most easily illustrated in cancer: tumors undergo a number of genetic changes that decrease the efficacy of administered drugs. siRNA therapies targeting these resistance pathways can be designed to improve outcomes, for example, by knocking out efflux proteins that pump chemotherapeutics out of the cell. Similarly, cancer cells that are affected by a small molecule inhibiting one pathway survive by reverting to another distinct pathway. Rational combination therapies could use siRNAs that knockdown the second pathway, and increase the likelihood the cancer cell will undergo apoptosis.

While a majority of the most advanced siRNA-delivery systems currently target the liver, there are innumerable patients that would benefit

from efficient delivery to nonliver tissues. It is likely that significant advances in delivery vehicles targeting almost every other tissue in the body will be required before the same clinical success is observed outside the liver. For example, there are already many well-known genetic diseases that affect the function of skeletal muscle (muscular dystrophies), cardiomyocytes (cardiomyopathies), and neurons (Huntington's disease). Many patients afflicted with these disorders would likely experience a dramatic improvement in their quality of life if appropriate delivery vehicles are discovered. However, testing for effective *in vivo* delivery using traditional molecular biology techniques can be time-consuming, difficult, and expensive. As a result, new assays designed to easily measure nonliver delivery in a meaningful way will need to be developed to efficiently screen for lead candidates.

Nonliver delivery may be further improved by a more complete understanding of the physiology that promotes disease. For instance, a number of neurological disorders are characterized by inflammatory signaling that results in dysfunctional and leaky vasculature. This pathological change may be used to differentially deliver siRNA to regions affected by disease. In the same way, diseases that result in differential metabolism and subsequent changes in lipid uptake may be targeted by conjugating siRNA to the lipids that are taken up by diseased cells. Although not all diseases will result in physiological changes that promote delivery, many new strategies for specific passive targeting may be uncovered by understanding disease physiology more completely.

Techniques that helped dramatically improve the efficiency of liver-targeting LNPs may be applied to next-generation conjugates targeting nonliver tissues. Many of the most successful LNP formulations were discovered by screening large numbers of compounds that were synthesized using high-throughput chemistry. These chemical synthesis schemes were robust, and did not require purification steps. As a result, thousands of materials could be synthesized with relative ease. By contrast, the chemistry required to conjugate different materials directly to siRNA has remained slow, complicated, and expensive. New chemical synthetic schemes will need to be developed so that large material libraries of conjugates can be easily synthesized and tested.

Advances in nanoparticle- and conjugate-based delivery systems are sure to affect the future of RNA-based medicine. Because RNAs can bind to nearly every type of biomolecule in a cell, the number of diseases that can be impacted by their regulation is likely to increase rapidly. As a result, delivery systems which deliver siRNA may be exploited to deliver other small

RNAs, while completely new systems may be required to deliver larger RNAs. In just over 10 years, our understanding of RNAs has made it clear that they will continue to play an increasingly important role in medicine, as long as we can deliver them safely *in vivo*.

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CHAPTER FOUR

Lipid Nanoparticles for Short Interfering RNA Delivery

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Contents

Abstract

The discovery of RNA interference (RNAi) in mammalian cells has created a new class of therapeutics based on the reversible silencing of specific disease-causing genes. This therapeutic potential depends on the ability to deliver inducers of RNAi, such as short-interfering RNA (siRNA) and micro-RNA (miRNA), to cells of target tissues. This chapter reviews various challenges and delivery strategies for siRNA, with a particular focus on the development of lipid nanoparticle (LNP) delivery technologies. Currently, LNP delivery systems are the most advanced technology for systemic delivery of siRNA, with numerous formulations under various stages of clinical trials. We also discuss methods to improve gene silencing potency of LNP-siRNA, as well as application of LNP technologies beyond siRNA to the encapsulation of other nucleic acids such as mRNA and clustered regularly interspaced short palindromic repeats (CRISPR).

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1. INTRODUCTION

Since the demonstration of RNA interference (RNAi) in mammalian model systems, much interest has arisen in the utilization of various promoters of RNAi, such as short-interfering RNA (siRNA) and microRNA (miRNA), for the treatment of diseases. RNAi is an endogenous process in eukaryotic cells that uses RNA molecules to catalyze degradation of specific, complementary messenger RNA (mRNA) sequences, possibly as a mechanism to protect against foreign pathogens such as viruses. When long double-stranded RNA is introduced into cells, a large, 200-kDa nuclease of the RNaseIII family known as Dicer (Filipowicz, Jaskiewicz, Kolb, & Pillai, 2005) cleaves the RNA into shorter fragments of approximately 21–23 nucleotides long. These fragments, or short-interfering RNA (siRNA) (Bernstein, Caudy, Hammond, & Hannon, 2001), are then loaded into the RNA-induced gene silencing complex (RISC) (Rand, Ginalski, Grishin, & Wang, 2004), a complex comprising many different proteins. One of these proteins is Argonaute 2 (Ago2), which is responsible for unwinding the siRNA and degradation of the sense or passenger strand (Matranga, Tomari, Shin, Bartel, & Zamore, 2005; Rand, Petersen, Du, & Wang, 2005). The single-stranded antisense strand then allows the RISC complex to actively seek out mRNA complementary to the antisense strand of the siRNA (Ameres, Martinez, & Schroeder, 2007). Once the mRNA is bound to the antisense strand of the siRNA, Ago2 mediates the cleavage of the mRNA between nucleotides 10 and 11 from the 5′ end of the antisense strand (Tomari & Zamore, 2005). The RISC complex can then seek out and degrade additional mRNA, leading to remarkable silencing of the target gene (Hutvágner & Zamore, 2002).

In theory, siRNA is able to specifically block the synthesis of any protein responsible for any disease; however, the actual translation of siRNA into clinical use has been hampered by several major difficulties. These include the need to protect siRNA from degradation by nucleases in biological fluids, the delivery of siRNA to target tissues, and the intracellular delivery of siRNA to the target cell's cytoplasm where RNAi takes place. Lipid nanoparticles (LNPs) are the leading delivery systems for enabling the therapeutic potential of siRNA for systemic applications (Semple et al., 2010; Zimmermann et al., 2006). LNP-siRNA systems containing optimized ionizable cationic lipids exhibit remarkable in vivo potencies in silencing liver (hepatocyte) target genes at doses as low as

0.005-mg siRNA/kg body weight following intravenous (i.v.) injection in rodents (Jayaraman et al., 2012; Semple et al., 2010). With therapeutic indices in mice approaching 1000, these systems are relatively nontoxic and demonstrate promising clinical utility. Here we review the challenges in siRNA delivery and different strategies to deliver siRNA, with a focus on LNP systems and their formulation methods. We also discuss the current LNP-siRNA formulations in clinical testing. It is important to note that technologies for the production of LNP systems are not restricted for siRNA but are also applicable to plasmid DNA, mRNA and potentially, clustered regularly interspaced short palindromic repeat (CRISPR) DNA. This versatility would not only pave the way for the translation of nucleic acid-based therapeutics into the clinic but would also expand our current arsenal of nucleic acid therapeutics.

2. CHALLENGES AND STRATEGIES FOR DELIVERY OF SIRNA

Delivery of siRNA for therapeutic purposes is hampered by several major hurdles. First, "naked" siRNA is unstable and rapidly degraded by nucleases in biological fluids (Choung, Kim, Kim, Park, & Choi, 2006; Layzer, Mccaffrey, Tanner, Huang, & Kay, 2004). It has been shown that chemical modifications of nucleic acids, such as 2′ *O*-methyl (Sproat, Lamond, Beijer, Neuner, & Ryder, 1989), 2′ fluoro-pyrimidine (Pieken, Olsen, Benseler, Aurup, & Eckstein, 2014), and phosphothioate linkages (Chowrira, & Burke, 1992) can protect siRNA from nuclease degradation. Second, siRNA cannot penetrate the cell membrane to enter the cytoplasm due to its large size, which ranges from 13 to 15 kDa, and negative charges (de Fougerolles, Vornlocher, Maraganore, & Lieberman, 2007; Whitehead, Langer, & Anderson, 2009). Moreover, despite evidence that naked siRNA can be taken up by endocytosis in neuronal cells, it faces an additional challenge of escaping the endocytosis-based degradation to mediate effective RNAi (Lingor, Michel, Schöll, Bähr, & Kügler, 2004). Finally, systemically administered siRNA accumulates in the liver and kidney and fails to accumulate at the site of disease (Braasch et al., 2004). As the glomerular filtration pore size is roughly 8 nm, naked siRNA are removed from circulation by the kidneys and excreted (Huang et al., 2011). Delivery vehicles are therefore necessary to overcome the above hurdles and realize the potential of siRNA as a therapeutic.

2.1 Localized Delivery of siRNA

Despite these challenges, accumulating evidence suggests that siRNA can be delivered locally to some tissues without the use of a sophisticated delivery system. One of the simplest strategies by which to administer unprotected, free siRNA for therapeutic purposes is directly to the localized tissue. It has been shown that intranasal administration of free siRNA against the P protein of the respiratory syncytial virus (RSV) and parainfluenza virus localizes siRNA to the lungs for up to 2 days and inhibits subsequent infections by these two viruses (Bitko, Musiyenko, Shulyayeva, & Barik, 2005). Furthermore, the siRNA shows effective antiviral properties when administered after RSV infection, with the best therapeutic response observed when the siRNA is administered at the time of viral infection (Bitko et al., 2005). Notably, the antiviral effects of this siRNA when complexed to the transfection reagent TransIT-TKO (Mirus Bio, Madison, WI) were significantly better than that of the naked form. A chemically modified siRNA targeting the nucleocapsid protein of RSV for intranasal delivery has since been developed by Alnylam Pharmaceuticals (Cambridge, MA). The siRNA formulation, named ALN-RSV01, was effective in inhibiting RSV when administered either prophylactically or therapeutically (Alvarez et al., 2009). In a phase II clinical trial, ALN-RSV01 was demonstrated to be safe and provided modest protection against RSV infection compared to placebo treatment (DeVincenzo et al., 2008, 2010). Another example of clinical applicability is the intranasally administered siRNA against the SARS coronavirus, which was shown to alleviate SARS-like symptoms in rhesus macaque (Li et al., 2005).

The eye is another popular tissue for local administration of siRNA. Age-related macular degeneration is a disease characterized by the development of choroidal neovascularization (CNV) in the eye. Intravitreal injection of naked siRNA against the vascular endothelial growth factor (VEGF) receptor has been shown to decrease the extent of CNV in mice (Shen et al., 2006). The siRNA was localized in the ganglion layer of the retina by 6 h after injection and the mRNA levels of VEGF receptor were significantly reduced by 7 days after injection. An intravitreal dose of as low as 0.5 μg siRNA was enough to suppress the development of laser-induced CNV in mice (Shen et al., 2006). In a similar study, intravitreal injection of siRNA targeting erythropoietin was effective at reducing neovascularization induced by hypoxia (Chen et al., 2009). In a nonhuman primate model of laser-induced CNV, an intravitreal dose of 70-μg siRNA targeting

VEGF suppressed the development of CNV for 36 days after laser induction (Tolentino et al., 2004).

The presence of the blood-brain barrier makes it extremely difficult for drugs, especially large molecules such as siRNA, to reach the central nervous system from the blood compartment. Direct administration is therefore the preferred route of drug entry. It has been shown that siRNA can be directly administered through either the intraventricular or intrathecal route to mediate gene silencing in the brain. Intraventricularly injected siRNA has been shown to mediate the gene silencing of enhanced green fluorescent protein (EGFP), dopamine transporter and serotonin transporter in the brain (Thakker et al., 2004, 2005). Also, intrathecally administered siRNA targeting the pain-related cation channel $P2X_3$ has been shown to reduce both $P2X_3$ expression and pain perception in treated mice (Dorn et al., 2004).

Despite the successes in the above examples, many tissues in the body remain inaccessible by local administration. Systemic delivery of drugs to these tissues would require the use of an appropriate delivery system.

2.2 Strategies for Systemic Delivery of siRNA

One of the simplest and earliest examples of systemic delivery of siRNA is the conjugation of cholesterol onto the 3′ end of the sense or passenger strand of the siRNA (Soutschek et al., 2004). Intravenous administration of cholesterol-conjugated siRNA against apolipoprotein B (apoB) effectively decreased levels of apoB mRNA in the liver as well as plasma apoB protein and serum cholesterol (Soutschek et al., 2004). Modifications such as 2′-*O*-methyl at the ribose and phosphothioate at the siRNA backbone offer general protection for the siRNA against hydrolysis by nucleases. Other modifications for siRNA are available and reviewed elsewhere (de Fougerolles et al., 2007; Kanasty, Dorkin, Vegas, & Anderson, 2013; Whitehead et al., 2009). The effective uptake of cholesterol-conjugated siRNA by hepatocytes is mediated via its association with serum lipoproteins (Wolfrum et al., 2007). Moreover, the reconstitution of cholesterol-conjugated siRNA into purified mouse high density lipoprotein (HDL) showed higher gene silencing efficacy than free cholesterol-conjugated siRNA (Wolfrum et al., 2007). Others also demonstrated that mimetic lipoprotein particles prepared from recombinant apolipoprotein A1 and apolipoprotein E3 can effectively deliver cholesterol-conjugated siRNA to the liver and mediate effective gene silencing (Nakayama et al., 2012). These examples highlight the importance of endogenous lipoproteins in

mediating the uptake of cholesterol-conjugated siRNA. Indeed, it was later revealed that LNP-siRNA also depends on the lipoprotein pathway for effective uptake in the liver (Akinc et al., 2010).

While cholesterol conjugation is a delivery strategy specifically designed for siRNA, most cationic polymers were initially used to deliver plasmid DNA or antisense oligonucleotides and later applied to siRNA. Polyethylenimine (PEI) is a polycationic polymer widely used for condensing nucleic acids into a transfection-competent polyplex (Baker et al., 1997; Utsuno & Uludağ, 2010; Zheng et al., 2012). It has been shown that polyplexes of PEI and siRNA targeting the *N*-methyl-D-aspartate (NMDA) receptor reduced the expression of the receptor and decreased pain perception in rats following intrathecal administration (Tan, Yang, Shih, Lan, & Cheng, 2005). After administration of 5 μg of siRNA, significant reduction in NMDA receptor mRNA was observed in 3 days and protein levels of the NMDA receptor was suppressed for 14 days (Tan et al., 2005). In addition to condensing nucleic acids, PEI has been shown to protect siRNA from degradation by serum nucleases (Kim et al., 2005; Mahato, Kumar, & Sharma, 2013; Scheule et al., 1997; Urban-Klein, Werth, Abuharbeid, Czubayko, & Aigner, 2005). PEI has also demonstrated efficacy through other means of administration. Intravenous administration of polyplexes of PEI and siRNA against VEGF has shown intratumoural localization of the polyplexes as well as silencing of VEGF in mice (Schiffelers et al., 2004). In a xenograft mouse tumor model, intraperitoneal injection of PEI-siRNA targeting the HER2 receptor led to the downregulation of HER2 expression and inhibition of tumor growth (Urban-Klein et al., 2005). Despite these successes, the toxicity of high molecular weight PEI, such as PEI87 (87 kDa) and PEI217 (217 kDa), remains a major hurdle for its clinical application (Thomas, Ge, Lu, Chen, & Klibanov, 2005; Thomas et al., 2005; Tseng, Mozumdar, & Huang, 2009; Zintchenko, Philipp, Dehshahri, & Wagner, 2008).

Cyclodextrin polymer (CDP) is another popular cationic polymer used for the delivery of siRNA in vivo. Each cyclodextrin monomer comprises five to eight glucopyranose molecules linked together into a ring by α1→4 linkages (Kanasty et al., 2013). These cyclodextrin monomers are then joined together by a linker containing cationic amidine groups, which bind nucleic acids including siRNA (Gonzalez, Hwang, & Davis, 1999). When mixed with plasmid DNA, CDP has been shown to self-assemble into a spherical complex of approximately 120 nm in diameter (Gonzalez et al., 1999; Hwang, Bellocq, & Davis, 2001). Interaction of CDP particles with plasma components can be prevented by coating the

particle surface with polyethylene glycol (PEG) conjugated to adamantane, which is a hydrophobic moiety that form an inclusion complex with cyclodextrin by inserting into the cyclodextrin core (Davis et al., 2004). Targeting ligands, such as transferrin, can be coupled to the adamantane-PEG to target the CDP nanoparticle to specific cells (Bellocq, Pun, Jensen, & Davis, 2003; Davis et al., 2004). In a mouse model of Ewing's sarcoma, transferrin-adamantane-PEG CDP nanoparticles were able to reduce metastatic tumor size after three consecutive daily doses of 2.5 mg/kg siRNA (Hu-Lieskovan, Heidel, Bartlett, Davis, & Triche, 2005). Although no measurable innate immune response or toxicity in the liver and kidney was observed, tumor volume rebounded soon after cessation of treatment (Hu-Lieskovan et al., 2005). In another study, CDP nanoparticles also did not show significant signs of toxicity or innate immune response in cynomolgus monkeys at doses up to 9 mg/kg siRNA; however, kidney and liver damage as well as elevated levels of pro-inflammatory cytokines were observed at a siRNA dose of 27 mg/kg (Heidel et al., 2007). Finally, in a phase I clinical trial involving melanoma patients, CDP nanoparticles containing siRNA against the ribonucleotide reductase M2 subunit (RRM2) were effective at reducing both the mRNA and protein levels of RRM2 in tumors (Davis et al., 2010). In addition to PEI and cyclodextrin, other synthetic polymers such as chitosan and poly(lactic-co-glycolic acid) are also under intensive investigation for their potential in siRNA delivery in vivo (Patil, Swaminathan, Sadhukha, Ma, & Panyam, 2010; Pillé et al., 2006; Yuan, Naguib, & Wu, 2011).

In comparison to cationic polymers, lipid nanoparticles (LNP) are currently the most mature technology enabling the delivery of siRNA in vivo, with at least four formulations of LNP-siRNA in various phases of clinical trials for the treatment of hypercholesterolemia, transthyretin-mediated amyloidosis and liver cancers (Allen & Cullis, 2013). LNP exhibit potent gene silencing activity in hepatocytes at doses as low as 0.005 mg siRNA/kg body weight in mice and 0.03 mg siRNA/kg in nonhuman primates following a single i.v. injection (Jayaraman et al., 2012). This is approximately 1000 times more effective than the examples of PEI and cyclodextrin delivery systems mentioned above. Furthermore, recent clinical results indicate that LNP-siRNA systems provide prolonged suppression of human transthyretin at a single dose of 0.3 mg siRNA/kg body weight, with full recovery occurring by day 70, the current gold standard for potency of a siRNA-based drug (Coelho et al., 2013). The following sections will discuss various formulation methods for these leading edge delivery systems.

3. LIPID-BASED DELIVERY SYSTEMS

Current development of the encapsulation technology for siRNA stems from early work on liposomes for small molecule delivery. In the early 1960s, Alec Bangham and colleagues realized that lecithin (egg yolk phosphatidylcholine) assembles into concentric lamellae when dispersed in water (Bangham & Horne, 1964). Later it was reported that these lipid vesicles are capable of maintaining a concentration gradient of ions, which can be disrupted by the addition of detergents (Bangham, Standish, & Watkins, 1965; Bangham, Standish, & Weissmann, 1965). Studies on the properties of lipid vesicles intensified soon after its discovery and it was quickly realized that the physical properties of these vesicles such as osmotic swelling and ability to trap solutes including ions and glucose were qualitatively similar to that of the cell membrane (Sessa & Weissmann, 1968). These lipid vesicles were initially termed "Bangasomes" due to their discovery by Alec D. Bangham; however, the term "liposome" was adopted subsequently (Sessa & Weissmann, 1968). In one of the first applications of liposomes as model biological membranes, Bangham and colleagues demonstrated that liposomes exposed to anesthetics were more permeable to ions (Johnson, Miller, & Bangham, 1973). Subsequently, liposomes have been widely used as drug delivery vehicles.

3.1 Lipid Systems for Small Molecule Drugs

The potential of the liposome as a drug carrier was recognized soon after its discovery in the early 1960s. Gregory Gregoriadis was one of the pioneers to recognize the drug delivery potential of liposomes and apply them in enzyme replacement therapy (Gregoriadis, Leathwood, & Ryman, 1971; Gregoriadis & Ryman, 1971). These systems were made by hydrating the dry lipid film with an aqueous buffer containing the enzyme amyloglucosidase or albumin. The resulting encapsulation efficiency was extremely poor, with less than 10% of the original enzymes and proteins being encapsulated. Encapsulation of antibiotics such as penicillin and actinomycin was also very poor (Gregoriadis, 1973).

The major breakthrough for improving drug encapsulation efficiencies in liposomes came with the development of the transmembrane pH gradient loading technique to entrap weakly basic drugs, which can be accomplished by three different methods (Figure 4.1). First, a pH gradient is generated by hydrating the lipid film with a low pH buffer followed by a buffer exchange

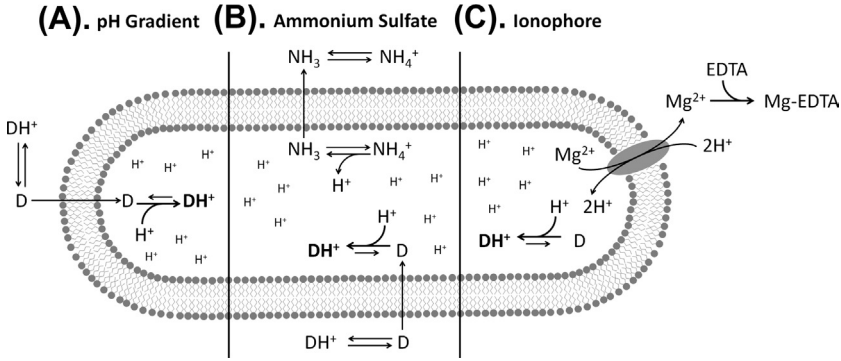

Figure 4.1 ***Encapsulation of small molecule drugs in response to transmembrane pH gradient.*** (A) In the standard pH gradient method, the lipid film is first hydrated with buffer of low pH (typically citrate buffer, pH 4), followed by extrusion generating large unilamellar vesicles. A pH gradient is generated by exchanging the external buffer with a buffer of higher pH (typically HEPES, pH 7.5). Weakly basic drugs enter the vesicle in their neutral form and then become protonated and trapped in the acidic interior of the vesicle. (B) In the ammonium sulfate method, a transmembrane ammonium sulfate gradient is first generated by hydrating and extruding the lipid in a buffer containing ammonium sulfate, followed by an exchange of the external buffer. The interior of the vesicle is acidified as the neutral NH_3 exits the vesicle, leaving behind a proton in the process. This pH gradient is then used for the encapsulation of weakly basic drugs. (C) In the ionophore method, a transmembrane gradient of Mg^{2+} or Mn^{2+} is first generated. The ionophore A23187 couples the export of Mg^{2+} with the import of two protons, therefore acidifying the interior of the vesicle. A chelator of Mg^{2+}, such as EDTA, is usually required to remove the Mg^{2+} as it is transported out of the vesicle.

using a solution of neutral pH (Figure 4.1(A)). It was demonstrated that weakly basic compounds such as catecholamines can be concentrated inside liposomes with an acidic interior (Nichols & Deamer, 1976). Later, it was shown that drugs that are weak bases, such as doxorubicin, could also accumulate in high amounts inside liposomes with an acidic interior (Mayer, Bally, & Cullis, 1986; Mayer, Bally, Hope, & Cullis, 1986). The encapsulation of drugs in response to a transmembrane pH gradient can be readily explained. At neutral pH, weakly basic drugs are a mixture of the protonated, membrane-impermeable form (DH^+) and the deprotonated, membrane-permeable form (D). The deprotonated form of the drug (D) can readily diffuse across the membrane and then becomes protonated (DH^+) and trapped inside the liposome where the pH is lower than the pK_a of the drug (Figure 4.1(A)). At equilibrium, the drug concentration gradient mirrors the proton gradient; thus a pH gradient of 3 units results in a 1000-fold higher drug concentration inside the liposome than that of the exterior medium.

In the second method, the loading of weakly basic drugs can be accomplished using ammonium sulfate (Bolotin et al., 1994). In this scenario, a pH gradient is generated by encapsulating ammonium sulfate inside the liposome followed by exchanging the external buffer (Figure 4.1(B)). An equilibrium of positively charged ammonium ion (NH_4^+) and neutral ammonia (NH_3) exists inside the liposome. Since the lipid membrane is highly permeable to the uncharged NH_3, one proton remains inside the liposome as NH_3 travels down its concentration gradient to the outside of the liposome, resulting in acidification of the liposome interior (Figure 4.1(B)). Loading of weak base drugs in response to pH gradient can result in interior drug concentrations that are so high that nanocrystals of drugs are formed, leading to a characteristic "coffee bean" appearance in electron micrographs (Abraham et al., 2005).

The use of an ionophore offers a third method to generate a pH gradient needed for the encapsulation of weakly basic drugs (Fenske et al., 1998). A transmembrane gradient of divalent cations such as Mn^{2+} or Mg^{2+} is first generated by preparing liposomes in solutions of $MnSO_4$ or $MgSO_4$ followed by an exchange of the external buffer with a sucrose solution. After generating the divalent cation gradient, the weakly basic drug, the ionophore A23187 and Ethylenediaminetetraacetic acid (EDTA) are added to the large unilamellar vesicle (LUV). The ionophore couples the transport of one divalent cation out of the LUV with the import of two protons, resulting in acidification of the vesicle interior (Figure 4.1(C)) (Fenske et al., 1998). The exported divalent cations are then chelated by EDTA while the drug enters the vesicles. This method showed very high levels of encapsulation (>80%) for both vincristine and ciprofloxacin with drug retention properties similar to other pH gradient loading methods (Fenske et al., 1998).

The goal of using liposomes as drug delivery vehicles is to enhance the potency of drugs and reduce their side effects by increasing their delivery to the sites of disease while avoiding the healthy tissues. Many studies have noted that the vasculature at sites of disease such as tumors, sites of infection and sites of inflammation is "leakier" than that of healthy tissues (Skinner, Tutton, & Brien, 1990; Steinberg, Konerding, & Streffer, 1990). For example, the neovasculature formed in tumors lacks the smooth muscle wall of normal vasculature and is relatively permeable to particles of 200-nm diameter or larger (Yuan et al., 1994, 1995). As a result, long-circulating liposomes with a diameter of 100 nm preferentially escape in the region of tumors, resulting in large increases in the amount of drug that is delivered to

the tumor. In addition, lymphatic drainage in the tumor is often impaired, causing liposomes to be retained in the interstitial space (Maeda, 2001). This combined phenomenon is called the enhanced permeation and retention (EPR) effect (Maeda, 2001; Maeda, Wu, Sawa, Matsumura, & Hori, 2000). It is important to note that the encapsulated drug is not available to target cells and must be released from the liposome to exert its effect. For certain drugs, the rate of drug release strongly influences the anticancer potency of the formulation. This is particularly true of cell cycle-specific drugs such as vincristine (Mayer et al., 1993; Webb, Harasym, Masin, Bally, & Mayer, 1995). To achieve optimal therapeutic activity, the rate of drug release can be controlled by changing the lipid composition of the liposome (Charrois & Allen, 2004) or by varying the drug-to-lipid ratio (Johnston et al., 2006).

Serum lipoproteins have been shown to play a critical role in both the circulation lifetime and cellular uptake of lipid nanoparticles. It has been shown that the apolipoproteins apoA-I, apoA-IV, and apoE bind to the surface of neutral phospholipid liposomes (Bisgaier, Siebenkas, & Williams, 1989). In particular, only apoE, but not apoA-I and apoA-IV, was shown to mediate cellular uptake of these liposomes by hepatocytes in vitro (Bisgaier et al., 1989). Similarly, apoE was shown to mediate hepatic uptake and blood clearance of neutral liposomes in vivo (Yan et al., 2005). ApoE is an apolipoprotein found on the surface of cholesterol-enriched lipoproteins, such as chylomicrons, very-low density lipoproteins (VDLs) and high density lipoproteins (HDLs) (Mahley, 1988; Mahley & Ji, 1999). The uptake of these lipoproteins by hepatocytes is mediated by receptor-mediated endocytosis of low density lipoprotein (LDL) receptor, LDL receptor (LDLR)-related protein and scavenger receptor BI (Beisiegel, Weber, Ihrke, Herz, & Stanley, 1989; Krieger, & Herz, 1994; Mahley & Ji, 1999). The role of apoE in mediating the hepatocellular uptake of LNP-siRNA system comprising ionizable cationic lipid was demonstrated in vivo with the use of ApoE-deficient mice (Akinc et al., 2010). The authors demonstrated that both ionizable LNP uptake by hepatocytes and silencing of the hepatic gene FactorVII were greatly reduced in ApoE-deficient mice compared to wild-type animals. The results were similar in *LDLR*$^{-/-}$ mice, which lack the LDLR to mediate hepatocellular uptake of the LNP. Interestingly, efficacy of LNP in ApoE-deficient mice can be rescued by conjugating the targeting ligand N-acetylgalactosamine (GalNAc) onto the PEG lipid of the LNP. GalNAc binds to the asialoglycoprotein receptor expressed on the surface of the hepatocyte with high affinity, providing an alternative pathway for LNP-siRNA to enter the hepatocytes of ApoE-deficient mice (Akinc et al., 2010). In contrast, LNP composed of a permanent positively charged cationic

lipid does not seem to depend on apoE for liver uptake, and its gene silencing efficacy in vivo was lower than that of ionizable LNP (Akinc et al., 2010).

3.2 Lipid Systems for siRNA

Unlike the entrapment of soluble drugs in neutral liposomes, cationic lipids are essential for efficient encapsulation of nucleic acids; lipid formulations without cationic lipids result in poor encapsulation of antisense oligonucleotides (Maclachlan, 2007). The use of cationic lipid was first described by Felgner and coworkers (Felgner et al., 1987), who used the permanently positively charged cationic lipid, *N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) to mediate cellular transfection of DNA. The positive charges of cationic lipids facilitate the encapsulation of anionic nucleic acids via electrostatic interactions. Cationic lipids can also provide a positive surface charge to the LNP, promoting close association of the LNP with the negatively charged cell surface. It has been shown that cell surface proteoglycan and sialic acids interact with cationic liposomes and facilitate their cellular uptake via the endocytic pathway (Mislick & Baldeschwieler, 1996; Mounkes, Zhong, Cipres-Palacin, Heath, & Debs, 1998; Stamatatos, Leventis, Zuckermann, & Silvius, 1988; Wrobel & Collins, 1995). Following endocytosis, cationic lipids of the LNP are hypothesized to form membrane disruptive ion pairs with anionic lipids of the endosomes, thereby facilitating intracellular release of nucleic acids (Hafez, & Cullis, 2001; Hafez, Maurer, & Cullis, 2001). However, positively charged liposomes are rapidly eliminated by the mononuclear phagocyte system (MPS) (Litzinger, 1997). Because most permanently charged cationic lipids are highly toxic, this has severely limited their in vivo application (Audouy, de Leij, Hoekstra, & Molema, 2002; Liebert et al., 2000; Scheule et al., 1997; Zhang, Liu, & Huang, 2005).

To avoid toxicity issues, ionizable cationic lipids with primary, secondary, or tertiary amines in the headgroup and apparent pK values of less than seven have been developed for the purposes of encapsulating nucleic acids when the lipid is positively charged at pH values below the pKa (e.g. pH 4), and for almost neutral LNP at physiological pH values. The first ionizable cationic lipid used for the encapsulation of nucleic acids was 1,2-dioleoyl-3-dimethylammonium propane (DODAP) (Maurer et al., 2001; Semple et al., 2001) with a pKa of 6.6 (Bailey & Cullis, 1994). Antisense oligonucleotides LNP containing DODAP exhibited a relatively long circulation lifetime following i.v. administration, as expected for a lipid particle with little surface charge (Semple et al., 2001).

In the past several years, significant improvement in the potency of LNP-siRNA was achieved by varying the chemical structure of the ionizable cationic lipid. This is noted in Figure 4.2 where the progression from DODAP to DODMA, DLinKDMA, DLinKC2DMA, and DLinMC3DMA is shown. These lipids were identified using an in vivo screening model employing LNP systems containing siRNA to silence Factor VII (FVII), one of the proteins in the blood-clotting cascade. Factor VII is made in hepatocytes and secreted into the circulation, thus the potency of the LNP-siRNA systems can be monitored by

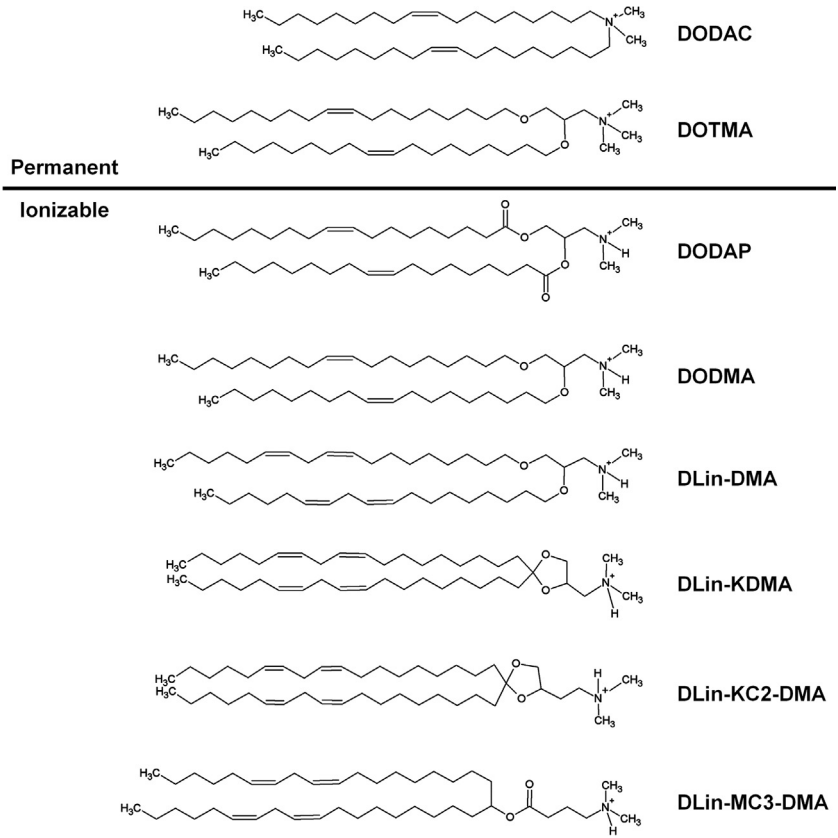

Figure 4.2 ***Evolution of cationic lipids.*** Early cationic lipids, such as DOTMA and DODAC, contain a permanently positive quaternary amine headgroup. Ionizable cationic lipids with tertiary amines in the headgroup have an apparent pKa less than seven, which allowed encapsulation of nucleic acids to be performed at acidic pH (pH 4.0) while the resulting LNP will exhibit a net neutral charge at physiological pH.

measuring FVII levels in the circulation 24 h after i.v. administration of the LNP. Remarkably, the potency of LNP-siRNA systems improved from ~10 mg siRNA/kg body weight to result in 50% gene silencing for LNP containing DODAP to 0.005 mg siRNA/kg body weight for the LNP containing DLinMC3DMA (Jayaraman et al., 2012; Semple et al., 2010). This improvement in potency is attributed to the use of ionizable cationic lipids with optimized pKa values and maximized bilayer destabilizing capabilities given the presence of anionic lipids that exist within the endosome. DLinMC3DMA, with a pKa of 6.4, is currently the most potent ionizable cationic lipid for hepatic gene silencing in vivo (Jayaraman et al., 2012).

The type of PEG lipid can also influence the potency of LNP-siRNA systems. PEG lipids are incorporated into LNP-siRNA formulations to prevent particle aggregation during the formulation process as the PEG moiety provides a hydrophilic steric barrier on the particle surface (Maurer, Fenske, & Cullis, 2001; Maurer et al., 2001). Without the PEG-lipid coating, it is impossible to produce small LNP-siRNA systems with diameters of less than 100 nm, and large micron sized aggregates are instead formed. Although the PEG coating is important for forming small and stable particles, it can also inhibit LNP association with the plasma membrane of target cells, indicating a need to remove the coating before cellular uptake can proceed. One potential solution is to use acid-labile PEG lipids where the PEG moiety dissociates from the lipid anchor at low pH (Choi, MacKay, & Szoka, 2003; Guo, & Szoka, 2001; Shin, Shum, & Thompson, 2003). However, the pH required is too low ($pH < 5$) to exist in the extracellular medium and thus the PEG lipids remain on the LNP surface, preventing close association with the cell membrane. Another approach is to use a PEG lipid that dissociates from the LNP in circulation following i.v. administration. The length of lipid chains anchored on the PEG lipid influences how long it stays on liposomes (Parr, Ansell, Choi, & Cullis, 1994), with longer acyl chains showing longer retention times than their shorter counterparts. Recently it has been shown that PEG lipids with short (C_{14}) acyl chains dissociate from LNP-siRNA in vivo with a half-time of approximately 1 h (Mui et al., 2013), whereas PEG lipids with longer (C_{18}) acyl chains exhibit dissociation rates of days or longer. This offers the possibility of employing LNP stabilized by PEG lipids with short acyl chains that rapidly dissociate following i.v. administration, allowing the LNP-siRNA systems to become potent transfection agents.

In addition to cationic lipids and PEG lipids, LNP formulations of siRNA typically contain other structural lipids. The most commonly used lipids are cholesterol and a saturated phosphatidylcholine (PC) such as distearoyl-PC (DSPC) or dipalmitoyl-PC (DPPC). The reasons as to why these lipids are required are unclear. Cholesterol is included because of historical data for bilayer liposomal systems indicating that liposomes consisting of PC alone accumulate cholesterol from serum components (Rodrigueza, Pritchard, & Hope, 1993); consequently, systems containing approximately equimolar cholesterol are in better equilibrium with their surroundings. The need for DSPC is counter-intuitive as the presence of DSPC would be expected to mitigate against fusion with the endosomal membrane following uptake into target cells due to its strong bilayer-stabilizing properties (Cullis, Hope, & Tilcock, 1986). However, recent data from our group suggested that elimination of DSPC compromises the gene silencing potency of LNP-siRNA systems for reasons yet to be determined (unpublished data).

There has been extensive work as well to incorporate targeting ligands into LNP systems. Targeting moieties such as antibody fragments and small molecule ligands have been coupled onto the surface of LNP in hopes of increasing cellular uptake of the LNP in target tissues through receptor-mediated endocytosis (Allen & Cullis, 2013; Sapra & Allen, 2003). It has been reported that liposomes with monoclonal antibody fragment against HER2 showed better cellular uptake in HER2-overexpressing breast cancer xenografts as compared to nontargeted liposomes (Kirpotin et al., 2006). Also, antibody-bearing liposomes containing antisense oligonucleotides against viral mRNA have showed better efficacy against viral infection compared to the same liposomes without the antibody (Leonetti, Machy, Degols, Lebleu, & Leserman, 1990). In addition to using antibodies as the targeting moiety, small molecules have also been coupled to the PEG lipid for enhancing LNP uptake in cells and subsequent gene silencing activity. For example, anisamide, a small compound that interacts with the sigma receptors, increases gene silencing activity of siRNA nanoparticles in lung tumors and metastases (Chen et al., 2009; Li & Huang, 2006; Li, Chono, & Huang, 2008). In addition, we have shown that strophanthidin, a cardiac glycoside that binds to the ubiquitously expressed cell surface receptor Na^+/K^+ ATPase, enhances delivery of LNP-siRNA to cells originating from a number of tissues such as the prostate, ovary, breast, lung, and pancreas (Tam et al., 2013). In many of these cases, the targeting moiety is conjugated to the distal end of the PEG lipid and the resulting targeting lipid is added along with the rest of the lipid components during the formulation process.

Alternatively, the targeting lipid is added to the stable, preformed LNP in a process called postinsertion (Iden & Allen, 2001). The use of postinsertion technique prevents the targeting lipid from interfering with the formulation process and provides a relatively simple method for preparing targeted LNP formulations.

3.3 Formulation Methods for LNP-siRNA

Similar to the development of cationic lipids, formulation technologies for siRNA were often designed for the delivery of plasmid DNA and later adopted for siRNA. The formulation used by Felgner and coworkers (Felgner et al., 1987) for cellular transfection was a simple mixture of DOTMA and dioleoyl-phosphatidylethanolamine (DOPE), which together form a complex (lipoplex) with plasmid DNA. Lipoplexes are often microns in diameter and are unstable and difficult to reproduce. They are often formulated with excess cationic charge not only to promote interaction with the plasmid DNA but also to facilitate association with the negatively charged cell surface and subsequent uptake by endocytosis (Stamatatos et al., 1988). Incorporation of helper lipids such as DOPE is thought to facilitate the release of plasmid DNA into the cytoplasm due to its propensity to adopt the nonbilayer, membrane lytic hexagonal H_{II} phase (Cullis & de Kruijff, 1978). Despite intensive efforts, however, lipoplexes have not proven to be useful for in vivo applications. The chief reason for this shortcoming is that they are highly toxic due to their positive charge and are quickly removed from the circulation by the fixed and free macrophages of the MPS due to their size and charge. As a result, they do not distribute efficiently to target cells such as tumor cells.

These difficulties have led to attempts to encapsulate nucleic acids within an enclosed bilayer membrane that exhibits low surface charge. In the "stabilized antisense lipid particle" method, a solution of the lipids in ethanol is added to an aqueous solution of nucleic acid at pH 4.0 (Semple et al., 2001). The relatively low pH of the buffer ensures that the ionizable cationic lipid, 1,2-dioleoyl-3-dimethylammonium propane (DODAP, pKa 6.6), is positively charged for interaction with the nucleic acid. The vesicles are subsequently extruded through polycarbonate membranes to ensure uniform size distribution. Dialysis in buffer at pH 7.0 removes residual ethanol in the mixture and deprotonates the ionizable cationic lipid, creating a relatively neutral system with prolonged circulation lifetimes (Figure 4.3). Encapsulation efficiencies of over 70% and particle diameter of about 100 nm were achieved with this method (Semple et al., 2001).

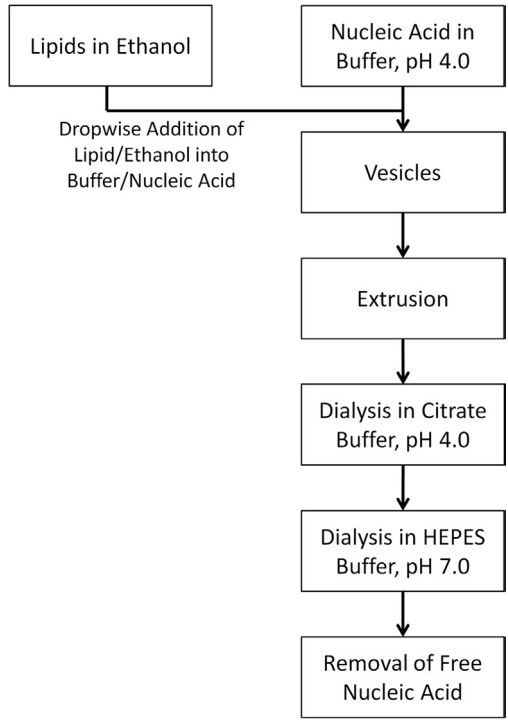

Figure 4.3 ***Encapsulation of nucleic acid by the "stabilized antisense lipid particle" method.*** This method involves the dropwise addition of an ethanolic solution of lipids into an aqueous buffer containing nucleic acid. The drop in ethanol concentration facilitates the formation of vesicle as the ethanol concentration is now below the solubility limit of the lipids. The vesicles are subsequently extruded, followed by dialysis to remove residual ethanol and to raise the pH of the buffer. Encapsulation efficiencies are typically in the range of 70%. The resulting particles are approximately 80–140 nm in diameter and show a range of lamellarity, depending on the initial nucleic acid-to-lipid ratio.

Interestingly, the resulting particles are mainly unilamellar at a low nucleic acid-to-lipid ratio, but adopt a multilamellar configuration at higher nucleic acid-to-lipid ratios. These observations led to the suggestion that nucleic acid bridges between individual lipid vesicles promoting the formation of multilamellar structures.

A variation of the ethanol-drop method is the preformed vesicle (PFV) method (Maurer et al., 2001). PFVs containing DODAP, cholesterol, DSPC and a PEG lipid at low pH, typically pH 4, where the DODAP is protonated, are prepared by extrusion in the presence of up to 40% ethanol (Figure 4.4). Extrusion is usually very rapid in the presence of ethanol.

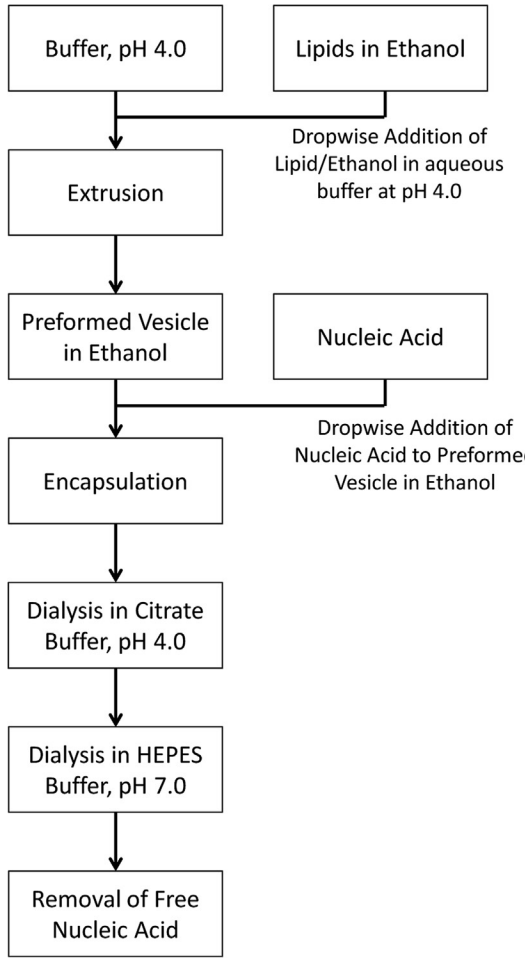

Figure 4.4 ***Encapsulation of nucleic acids by the preformed vesicle method.*** Lipid vesicles are first produced by dropwise addition of an ethanolic solution of lipids into an aqueous buffer, followed by extrusion. An aqueous solution of nucleic acid is added slowly to the resulting vesicle solution, which usually contains approximately 40% ethanol. The mixture is incubated for 1 h at 37 °C to allow encapsulation to take place. Residual ethanol is removed by dialysis and the pH of the solution is raised to 7.0. This method usually results in multilamellar particle of approximately 100 nm in diameter with an encapsulation efficiency of approximately 80%.

A solution of nucleic acid at pH 4.0 is then slowly added to the ethanol-destabilized liposomes to avoid particle aggregation and the mixture is then incubated for 1 h to allow encapsulation of the nucleic acid. Ethanol is removed by dialysis after the encapsulation step and the pH of the solution

is adjusted to 7.0 to create a charge neutral system. This method generated multilamellar particles of approximately 100 nm in diameter with nucleic acid encapsulation efficiency of over 80% (Maurer et al., 2001). It was suggested that the attachment of nucleic acid onto the surface of the extruded liposome creates an adhesion point between liposomes, facilitating the formation of the multilamellar vesicles. This also explains the high nucleic acid encapsulation efficiency achieved with this method as the nucleic acid appears to be entrapped between lamellae of the multilamellar particle.

An alternative method to extrusion is the spontaneous vesicle formation (SVF) by ethanol dilution method (Jeffs et al., 2005). Similar to many methods of encapsulation, this method was first developed for plasmid DNA and later adopted for the encapsulation of siRNA. In this method, nucleic acid is prepared in an acidic buffer, while cationic lipid, DSPC, cholesterol and PEG lipid are dissolved in ethanol. The nucleic acid and lipid mixtures are then combined using a T-tube mixer, with the flow of the two streams of fluid controlled via a peristaltic pump (Figure 4.5) (Jeffs et al., 2005). Vesicles form spontaneously as the lipids precipitate from solution as the polarity of the medium is raised. Residual ethanol in the final mixture is then removed by dialysis. For siRNA encapsulation, this method uses low amounts of PEG lipid, typically in the range of 1–5% (mol% lipid), and results in particles ranging from 70 to 80 nm in diameter (Judge, Bola, Lee, & MacLachlan, 2006; Zimmermann et al., 2006). The encapsulation of siRNA is highly efficient with efficiencies of over 90% routinely achieved. The resulting LNP-siRNA are termed "stable nucleic acid lipid particles". Limitations of T-tube mixer formulation include the difficulty in applying the process to laboratory scale formulations due to the high flow rates needed to achieve rapid mixing, and the limited mixing rate which results in an inability to formulate LNP with lipid compositions that require very rapid mixing to achieve stable systems.

The Cullis laboratory has developed an in-line mixing method employing a microfluidic herring-bone micromixer (Belliveau et al., 2012). The herring-bone micromixer has two inlets, one for an ethanolic mixture of lipids and the other for a buffered solution of siRNA at pH 4.0. A dual syringe pump is used to drive the two streams of fluid into the herring-bone micromixer (Figure 4.6). As the two streams of fluid meet at the herring-bone micromixer, they fold and wrap around each other, exponentially decreasing the diffusion length between the two streams. This allows for rapid mixing of the two streams of fluid on a millisecond timescale. The rapid decrease in solvent polarity results in the precipitation of lipids in accordance to their solubility.

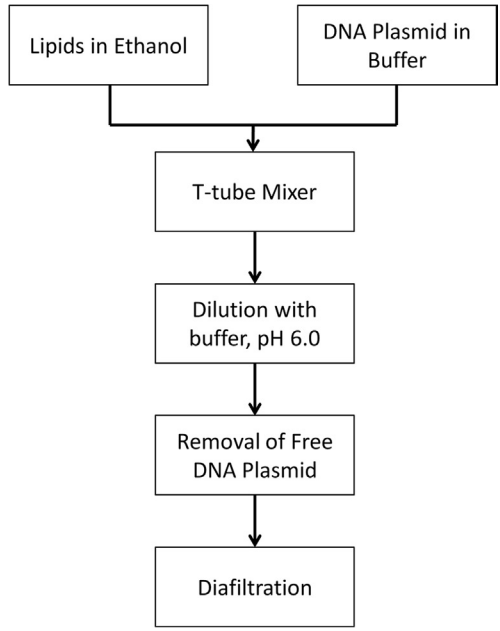

Figure 4.5 ***Encapsulation of nucleic acid with spontaneous vesicle formation by ethanol dilution.*** This method is also commonly referred as the "T-tube" method. It involves mixing of lipid components dissolved in ethanol with nucleic acid in aqueous buffer using a T-tube connector. The flow of the two streams of fluid is controlled with a peristaltic pump. Particles are formed as the ethanol is diluted below the solubility limit of the lipid. The ethanol content is further diluted upon exiting the T-tube connector to stabilize the resulting particles. Unencapsulated nucleic acid can be removed by using an ion exchange column. Residual ethanol is removed by diafiltration against phosphate buffered saline (PBS). This method has been used for encapsulation of plasmid DNA (stabilized plasmid-lipid particles), antisense oligonucleotides and siRNA (stable nucleic acid lipid particle).

This technique has resulted in greater than 90% siRNA encapsulation efficiency over a wide range of siRNA-to-lipid ratios (Leung et al., 2012). Cryo-transmission electron microscopy has indicated that LNP-siRNA produced by microfluidic mixing exhibit an electron dense core similar to the ones produced by T-tube in-line mixing (Figure 4.7). Additional biochemical characterizations strongly suggest that the solid, electron dense core is comprised of distorted inverted micelles of cationic lipids complexed to the encapsulated siRNA. Furthermore, molecular dynamics simulation of LNP-siRNA systems suggests the particles contain a nanostructured, hydrophobic core with the siRNA surrounded by an inverted micelle of ionizable cationic lipids (Figure 4.8). These results are consistent with the initial condensation of

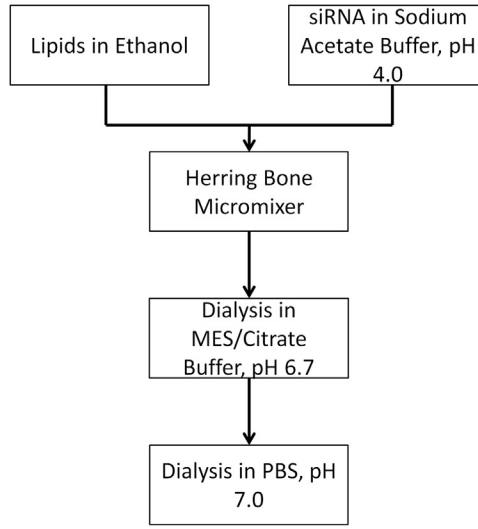

Figure 4.6 ***Encapsulation of siRNA by microfluidic mixing.*** This method employs a herringbone micromixer to facilitate the mixing of lipid components dissolved in ethanol and siRNA in aqueous buffer. The flow of the two streams of fluid is controlled using a dual syringe pump. The herringbone micromixer exponentially increases the surface area between the two streams of fluid, resulting in rapid mixing on a millisecond timescale. Residual ethanol is removed by dialysis and the pH of the solution is raised to 7.0. This method results in over 90% siRNA encapsulation efficiencies. Particle size is adjustable from 20 to 50 nm by varying the PEG-lipid content from 5% to 1%.

inverted micelles of ionizable cationic lipids around the siRNA, which serve as nucleating structures for the rest of the lipids to assemble into a solid core LNP (Figure 4.9). The most hydrophilic lipid, the PEG lipid, would be the last component to be deposited on the nascent LNP thus providing an outer shell for the stabilized particle (Figure 4.9). The results are also consistent with the PEG lipid, as the last component to be deposited on the nascent LNP, providing the outer shell of the stabilized particle (Figure 4.8). Based on this model, one would expect the size of the resulting LNP to be dictated by the ratio of "core" lipid to "surface" lipid in the lipid mix. Indeed, the size of the particle is freely adjustable between 20 and 100 nm in diameter simply by altering the PEG-lipid content of the formulation (Belliveau et al., 2012). In comparison with the PFV technique, microfluidic mixing results in higher encapsulation efficiency (>90%), generates smaller particles, and permits small scale production with little loss due to dead volume. This method allows for well-defined and reproducible mixing between the solutions of lipids and siRNA, resulting in lower batch-to-batch variation (Belliveau et al., 2012).

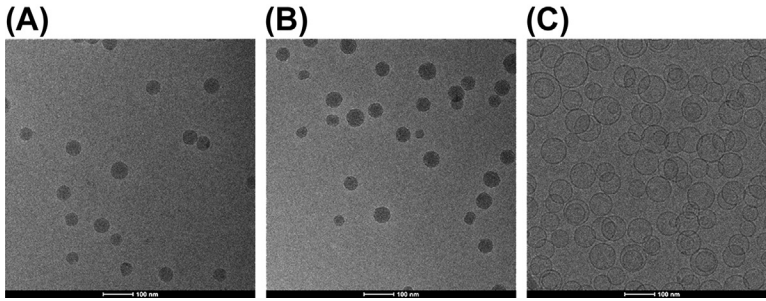

Figure 4.7 ***Cationic LNP produced by microfluidic mixing exhibit electron dense cores both in the presences and absence of siRNA as indicated by cryo-TEM.*** (A), Cryo-TEM micrograph obtained from siRNA-LNP with the lipid composition cationic lipid/DSPC/Cholesterol/PEG lipid (40/11.5/47.5/1; mol/mol) at a siRNA/lipid ratio of 0.06, wt/wt. (B), LNP with the same lipid composition as (A) but prepared in the absence of siRNA. (C), POPC/cholesterol (50/50; mol/mol) bilayer vesicles prepared by extrusion through polycarbonate filters with 80 nm pore size. *Reproduced with permission from Leung et al. (2012).*

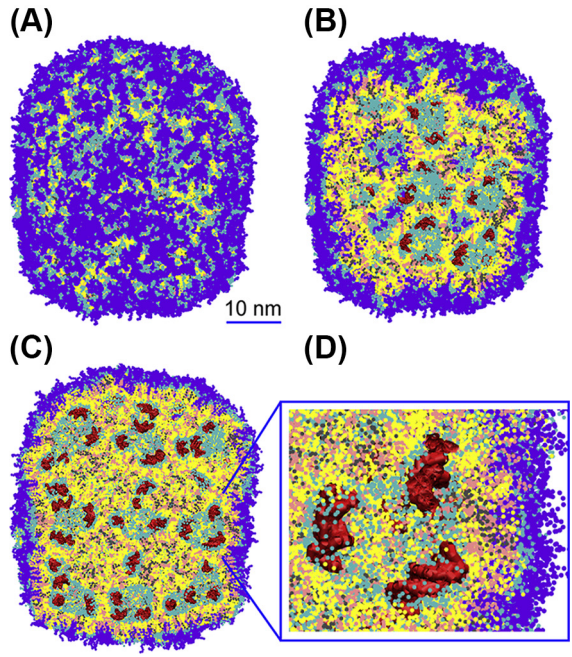

Figure 4.8 ***A lipid nanoparticle (LNP) contains irregular water-filled cavities separated by bilayer membranes, with nucleic acids bound to the membrane surface.*** (A) side view, (B,C) cross-section, and (D) zoom-in views. Cationic lipid DLin-KC2-DMA is shown in yellow, cholesterol in pink, DSPC in grey, lipid polar moiety in cyan, PEG lipid in violet, nucleic acids (duplex DNA) in red, water not shown for clarity. The lipid composition was DLin-KC2-DMA/DSPC/cholesterol/PEG lipid (4:1:4:1; mol/mol) and DNA to lipid ratio ~0.05 wt/wt. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.) *Reproduced with permission from Leung et al. (2012).*

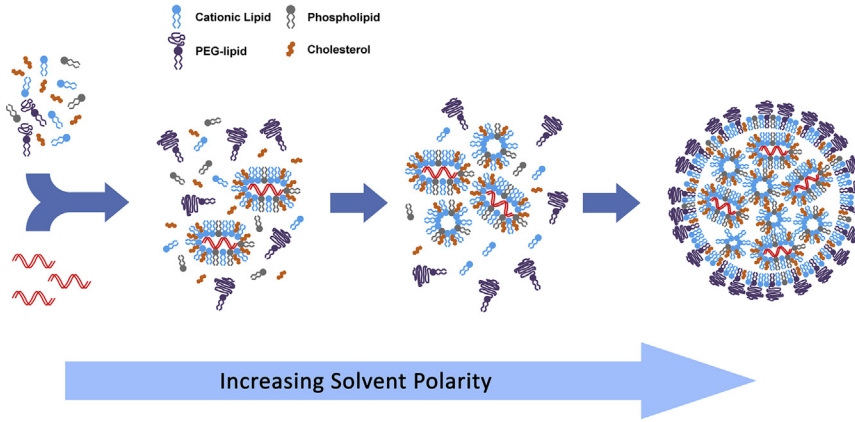

Figure 4.9 ***Assembly of LNP-siRNA by microfluidic mixing.*** Lipid components reach their individual solubility limit and precipitate out of solution as the ethanol in the lipid stream is being diluted by the aqueous stream. The acidic sodium acetate buffer protonates the ionizable cationic lipids (blue), which then form an inverted micelle around the siRNA (red) via electrostatic interaction. As the polarity of the solvent increases, inverted micelles begin to aggregate, which is followed by coating by PEG lipids (purple) to form the LNP-siRNA. (See the color plate.)

The above-mentioned examples of formulation rely on ionizable cationic lipids to condense the siRNA for encapsulation into LNP. Alternatively, a lipid-coated calcium phosphate (LCP) nanoparticle that utilizes calcium phosphate to condense siRNA before encapsulation by a lipid layer was developed (Li, Chen, Tseng, Mozumdar, & Huang, 2010). The fabrication process involves combining calcium chloride, sodium phosphate, and siRNA in a cyclohexane/Triton-X-100/hexanol solution to form a reversed-phase, water-in-oil micro-emulsion (Figure 4.10). Aqueous sodium citrate is then added to the emulsion to form calcium phosphate precipitates with the entrapped siRNA. The calcium phosphate precipitate is then purified using silica gel and combined with liposomes made of the permanently charged cationic lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and cholesterol to form the LCP nanoparticle (Figure 4.10). This LCP formulation was later modified to include the anionic lipid dioleoyl-phosphatidic acid (DOPA), which serves as an inner leaflet lipid to coat the calcium phosphate precipitate (Li, Yang, & Huang, 2012). Electron micrographs of DOPA coated calcium phosphate showed a hollow core of approximately 20 nm in diameter. This is consistent with the hypothesis that the negatively charged DOPA headgroup interacts with the positively charged calcium phosphate core in a way that the acyl chains of DOPA face the exterior of the particle, forming an inverted micelle-like structure.

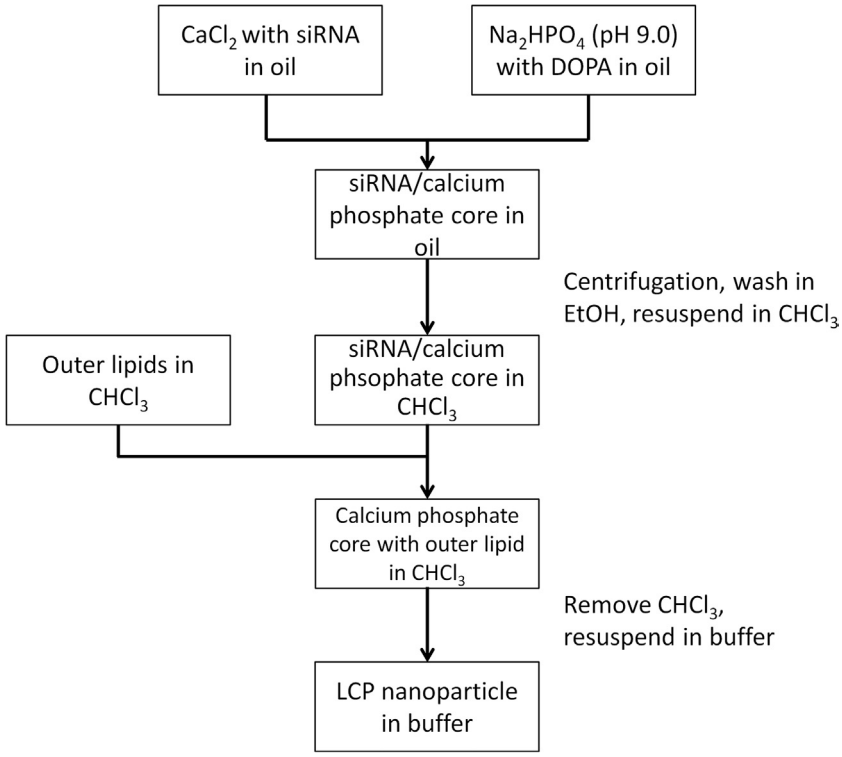

Figure 4.10 ***Preparation of lipid-coated calcium phosphate (LCP) nanoparticle.*** This method consists of first preparing a solution containing calcium chloride and siRNA dispersed in a cyclohexane/Igepal oil, as well as a solution of dioleoyl-phosphatidic acid (DOPA) and sodium phosphate dibasic (pH 9) similarly dispersed in a cyclohexane/Igepal oil. These two solutions are then mixed for 20 min and the calcium phosphate cores containing the siRNA are isolated by centrifugation followed by a wash with ethanol. A mixture of outer lipids containing DOTAP, cholesterol and PEG lipid are added to the cores resuspended in chloroform. The chloroform is later removed by evaporation and the resulting LCP nanoparticle solution is resuspended in aqueous buffer.

The surface of the particle is then coated with a variety of surface lipids to adjust the surface charge of the final LCP from positive to negative. In addition, PEG lipids can be included to prolong the circulation lifetime of the resulting LCP nanoparticle (Li et al., 2012). siRNA encapsulation efficiency of the LCP nanoparticle is typically over 90% with a particle size of approximately 40 nm in diameter including the PEG coating (Li et al., 2012). An LCP nanoparticle constructed with the cationic lipid DOTAP and an anisamide targeting PEG lipid has demonstrated efficacy in inhibiting lung metastasis in a melanoma mouse model (Yang, Li, Liu, & Huang,

2012). This nanoparticle contains three different siRNA sequences targeting MDM2, c-myc, and VEGF in order to achieve a greater therapeutic effect by inhibiting multiple pathways simultaneously. Mice treated with this targeted LCP particle have shown significantly improved survival rates and have markedly reduced numbers of lung metastatic nodules compared to either control mice or mice treated with LCP without the anisamide targeting ligand (Yang et al., 2012). In addition, the LCP technology has been used to co-encapsulate siRNA with gemcitabine, a nucleoside analog used as a first-line chemotherapeutic for advanced non-small cell lung carcinomas (Zhang, Schwerbrock, Rogers, Kim, & Huang, 2013). The mechanism of action of LCP nanoparticle is still under investigation; however, it is hypothesized that the acidic environment inside the endosome dissolves the calcium phosphate core of these particles, which causes an increase in the endosomal osmotic pressure that eventually breaks the endosome and release the siRNA into the cytoplasm (Li et al., 2010). It is important to note that divalent cations such as calcium and magnesium are known to induce membrane-disrupting hexagonal H_{II} phases in membranes containing anionic lipids, such as the ones found in the endosomal membrane (Hope, Walker, & Cullis, 1983; Tilcock, & Cullis, 1981). It is possible that the calcium released from the LCP particle induces hexagonal H_{II} phases with anionic lipids of the endosomal membrane, thereby disrupting the integrity of the endosomal membrane and facilitating the intracellular release of the encapsulated siRNA.

4. LNP-SIRNA FORMULATIONS IN CLINICAL TRIALS

Recent advances in lipid-based formulation technologies have led to numerous LNP-siRNA formulations in various stages of clinical trials. One of the most advanced formulations is ALN-TTR02 from Alnylam Pharmaceuticals (Cambridge, MA), which is currently in a phase III clinical trial for the treatment of transthyretin (TTR) amyloidosis. In nonhuman primates, ALN-TTR02 was shown to cause a 75% decrease in serum TTR levels 7 days after a single i.v. dose of 0.1 mg siRNA/kg body weight (Coelho et al., 2013). For animals receiving a single 0.3 mg/kg dose of siRNA, over 70% suppression of serum TTR levels was observed for 28 days after LNP-siRNA injection. Similarly in humans, the authors observed 50% knockdown of TTR levels by day 3 after a single 0.3 mg/kg dose of siRNA and over 50% reduction in protein levels for 28 days (Coelho et al., 2013). The suppression of serum TTR levels by ALN-TTR02 was specific as siRNA

targeting an unrelated protein had no effects on serum TTR levels. Furthermore, the formulation was well tolerated, with only minor infusion-related reactions and no liver and kidney toxicity.

Another promising formulation from Alnylam Pharmaceuticals is ALN-PCS, which is designed to specifically target proprotein convertase subtilisin/kexin type 9 (PCSK9) produced by liver for the treatment of hypercholesterolemia. Mice lacking PCSK9 was shown to have increased expression of the LDLR in the liver and decreased levels of cholesterol in the plasma (Rashid et al., 2005). Knockdown of PCSK9 in nonhuman primates by LNP-siRNA was shown to lower serum LDL cholesterol levels and increase the expression of LDLR in the liver (Frank-Kamenetsky et al., 2008). In a recent phase I clinical trial, a single dose of ALN-PCS at 0.4 mg siRNA/kg body weight caused a 70% decrease in serum PCSK9 levels 3 days after injection (Fitzgerald et al., 2014). This has consequently resulted in a 40% reduction in serum LDL cholesterol level that required 30 days to recover to the pre-treatment level. Similar to the ALN-TTR02 formulation, ALN-PCS was well tolerated.

In addition to targeting intrinsic proteins of the body, the same LNP-siRNA technology is also actively pursued as a therapeutic against viral infections. Tekmira Pharmaceuticals (Burnaby, BC) has recently launched a Phase I clinical study for TKM-Ebola, an LNP-siRNA formulation for the treatment of infection from the Zaire strain of Ebola virus (ZEBOV). This formulation was produced using the T-tube in-line mixing technique mentioned above and contained three different siRNA sequences targeting the L polymerase, viral protein 24 and viral protein 35 of ZEBOV (Geisbert et al., 2010). In a preclinical study utilizing nonhuman primates, all animals challenged with ZEBOV by intramuscular inoculation survived the infection following seven daily doses of TKM-Ebola at 2 mg siRNA/kg body weight per dose. The investigators were unable to detect ZEBOV in any of the treated animals after 14 days. Control animals treated with LNP-siRNA formulated with an unrelated siRNA against luciferase succumbed to the viral infection 10 days after viral inoculation. TKM-Ebola was granted Fast Track designation from the U.S. Food and Drug Administration (FDA) in March 2014.

Although LNP-siRNA systems are currently the most advanced siRNA delivery technology in clinical trials, lipoplexes of lipid and siRNA are also under active clinical investigations as means to deliver therapeutic siRNA in vivo. One such formulation is Atu027 from Silence Therapeutics GmbH (Berlin, Germany), a lipoplex formulation targeting protein kinase N3

(PKN3) for the treatment of advanced solid tumors (Aleku et al., 2008). This formulation contains a polycationic lipid, AtuFECT01, and is prepared by a method very similar to the PFV method described above (Santel et al., 2006). One very important difference from the PFV method is the lack of ethanol during the formulation process. Ethanol is an important factor in facilitating the encapsulation of siRNA as it allows remodelling of PFVs into multilamellar vesicles after membranes are bridged together by electrostatic interactions with the siRNA (Maurer et al., 2001). Without the ethanol, the formulation is likely a complex of cationic liposomes linked together by siRNA on the surface. Regardless of the formulation procedure, Atu027 was effective at silencing PKN3 mRNA levels in the lung and liver of mice following tail vein injections of four daily doses of Atu027 at 0.7 mg siRNA/kg per dose (Aleku et al., 2008). The formulation was successful at reducing the volume of xenografted tumor in the prostate and lowering the amount of metastatic tumors in the lymph node of mice. The company is currently conducting Phase Ib/IIa clinical trials of Atu027 in combination with gemcitabine for the treatment of advanced pancreatic cancer. Interestingly, localization of Atu027 to the lungs following i.v. administration has prompted the company to develop a lipoplex formulation, DACC, to treat lung metastases (Fehring et al., 2014). Effective reduction of CD31 expression in lung tumors and an increased survival rate were observed in a mouse model of lung metastases injected via tail vein with DACC complexed with siRNA against CD31 (Fehring et al., 2014). It remains to be seen whether this formulation would be advanced to clinical testing for the treatment of lung metastases.

5. FUTURE PROSPECTS

5.1 Avenues for Improving LNP Delivery

As mentioned above, tremendous efforts have been invested in the design of ionizable cationic lipids for maximizing the gene silencing capability of LNP-siRNA (Jayaraman et al., 2012; Semple et al., 2010). Although hepatic gene silencing resulted from LNP-siRNA containing the new generation of ionizable cationic lipids was greatly improved as compared to systems made with earlier generations of lipids, a recent study suggested that the intracellular release of siRNA from the current LNP-siRNA systems is a highly inefficient process. Following uptake of LNP-siRNA into the cell via endocytosis, only 1–2% of the total siRNA is released into the cytosol within a small window of time when LNP-siRNA accumulate in a specific

endocytic compartment with both early and late endosomal characteristics (Gilleron et al., 2013). In addition, the authors did not detect any major disruption of the endosomal membrane. Another recent report on the intracellular trafficking of LNP-siRNA systems revealed that approximately 70% of internalized particles eventually exits the cell through a recycling mechanism dependent on the protein Niemann-Pick Type C-1 (NPC1) (Sahay et al., 2013). Cells lacking the NPC1 gene were shown to have reduced endosomal recycling activities between late endosomes or lysosomes to the extracellular medium. As a result, these cells retain more LNP-siRNA following endocytosis and show more potent gene silencing than wild-type cells. It is therefore possible to improve the gene silencing potency of LNP-siRNA systems by manipulating the endocytic pathways to increase the intracellular accumulation of LNP-siRNA and/or the release of siRNA into the cytosol. Coincidentally, cellular entry of Ebola virus also requires functional NPC1 and treatment of cells with small molecular inhibitors of NPC1 greatly attenuates infections by the virus (Carette et al., 2011; Côté et al., 2011). Such small molecule inhibitors of NPC1 can be used in conjunction with LNP-siRNA to inhibit LNP-siRNA recycling and thus enhance the bioavailability of the internalized siRNA and gene silencing potency.

Incorporation of metallic nanoparticles in LNP-siRNA systems may offer an attractive method to disrupt the endosomal membrane. By exciting the metallic nanoparticle embedded in a lipid membrane through light radiation or a magnetic field, it is possible to increase the temperature of the lipid membrane to its gel-to-liquid crystal phase transition temperature (T_M) at which the permeability of the membrane is the greatest. Liposomes with gold nanoparticles embedded in their membrane have been shown to release their aqueous content following irradiation with ultra-violet light (Paasonen et al., 2007). In addition to gold, other metallic nanoparticles such as iron oxide were utilized in generating thermo-sensitive liposomes (Amstad et al., 2011). Formulations of gold nanoparticles with liposome have been prepared by several different methods: (1) reverse-phase evaporation with gold nanoparticles suspended in the organic phase (Hong, Friend, Glabe, & Papahadjopoulos, 1983; Paasonen et al., 2007); (2) physical adsorption of gold nanoparticles onto the surface of liposomes (Kojima, Hirano, Yuba, Harada, & Kono, 2008); (3) postinsertion of gold nanoparticle-conjugated phospholipids onto preformed liposomes (Chithrani, Dunne, Stewart, Allen, & Jaffray, 2010) and (4) embedding gold nanoparticle in a dry lipid film followed by hydration (Park, Oh, Mun, & Han, 2006). Recently, our laboratory

has demonstrated encapsulation of gold nanoparticles in LNP by mixing the commonly used lipids in LNP-siRNA formulation with commercially available negatively charged gold nanoparticles in a microfluidic mixer (unpublished). This method is straightforward as the ionizable cationic lipids bind to the negatively charged gold nanoparticles in a manner similar to the encapsulation of siRNA. Using the same method, LNP encapsulation of iron oxide nanoparticles is potentially possible when they are modified with functional groups such as carboxylic acids or silica to render their surface negatively charged (Laurent et al., 2008). A thermo-sensitive LNP-siRNA system can be generated by co-encapsulating metallic nanoparticles with siRNA using ionizable cationic lipids. Enhanced release of siRNA following cellular uptake may be made possible by excitation of cells or tissues with ultra-violet light or alternating magnetic field to generate enough heat to disrupt or permeabilize the endosomal membrane.

In addition to the potential benefit of enhancing endosomal release of siRNA, metallic nanoparticles co-encapsulated with siRNA in the same LNP system can also serve as a contrast agent for bioimaging. Gold nanoparticles, a good absorber of X-ray radiation, have been shown to provide therapeutic effects in tumor-bearing mice after radiotherapy and enhance contrast of tumors during X-ray imaging (Hainfeld, Slatkin, & Smilowitz, 2004; Hainfeld, Slatkin, Focella, & Smilowitz, 2006; Hainfeld, Smilowitz, O'Connor, Dilmanian, & Slatkin, 2013). However, the use of gold nanoparticles in radiation medicine or bioimaging suffers from poor accumulation of gold nanoparticles in tumors and rapid excretion through the kidneys following i.v. injection (Hainfeld et al., 2004). Thus, a LNP carrier system has the potential to improve the accumulation of the metallic nanoparticle to tumor sites by the aforementioned EPR effect. Co-encapsulation of siRNA with metallic nanoparticles can therefore generate an LNP system with dual functionality that allows both bioimaging of the tumor and silencing of the target gene for therapeutic purposes. It is anticipated that such "theranostic" system would have tremendous impact in the biomedical applications of LNP technologies.

5.2 Delivery of Other Nucleic Acids

The ionizable cationic lipid-based encapsulation technology for siRNA described above is fully applicable to other nucleic acid polymers such as plasmids and mRNA. A major challenge for LNP-plasmid systems is sufficient target gene expression since the nuclear envelope remains the barrier to the translocation of plasmid DNA into the nucleus. Efficient transgene

expression is usually only observed in rapidly dividing cell where the nuclear envelope disintegrates during mitosis allowing the plasmid DNA to enter the nucleus (Bally et al., 1999; Mortimer et al., 1999). In contrast, mRNA-LNP systems eliminate the need for nuclear translocation for gene expression (Hecker, 2013; Zou, Scarfo, Nantz, & Hecker, 2010). It has been shown that mRNA delivered by electroporation can result in the expression of target gene (Van Tendeloo et al., 2001). The therapeutic potential of mRNA was demonstrated by the fact that chemically modified mRNA can mediate the expression of the target protein, SP-B, in the lungs following intratracheal administration in SP-B deficient mice (Kormann et al., 2011). However, many tissues are inaccessible by local administration and the LNP-mRNA systems should allow systemic delivery with particular potency for tissues such as the liver (hepatocytes). Recently, it has been demonstrated that chemically modified mRNA condensed by protamine can be encapsulated inside of preformed cationic vesicles (Wang et al., 2013). The microfluidic mixing method presented previously would eliminate the need for nucleic acid-condensing agents such as protamine and the need for the time-consuming extrusion process. As LNP-mRNA systems produced by microfluidic mixing would contain the same lipid components as used for the delivery of siRNA, it is expected that these LNP-mRNA would display biodistribution and pharmacokinetic properties similar to that of LNP-siRNA systems. The ability to systemically deliver mRNA has considerable potential for the treatment of cancer and a variety of genetic disorders.

Another genetic material that can benefit from lipid-based delivery for therapeutic purposes is the recently discovered clustered, regularly interspaced, short palindromic repeats (CRISPR). CRISPR were originally discovered in *Escherichia coli* and was subsequently recognized as an important adaptive immunity pathway found in bacteria and archaea (Ishino, Shinagawa, Makino, Amemura, & Nakata, 1987; Marraffini, & Sontheimer, 2010). The mechanism of bacterial adaptive immunity by CRISPR has been reviewed extensively elsewhere (Marraffini, & Sontheimer, 2010; Rusk, 2012; Sorek, Kunin, & Hugenholtz, 2008; Waters & Storz, 2009; Wiedenheft, Sternberg, & Doudna, 2012). Recent interests in CRISPR technology stemmed from the possibility of using CRISPR to modify genes in order to tackle human diseases. CRISPR, along with the endonuclease CRISPR-associated protein 9 (Cas9), facilitate genome editing by introducing double-strand breaks at a specific genetic locus that has sequence complementary to the guide RNA (gRNA) (Jinek et al., 2012, 2013). A gene can therefore be deleted

or replaced using this novel technology. The CRISPR system has been used to produce genetically modified animals from numerous species, including fruit fly, zebrafish, rabbit, rat and mouse (reviewed in Sander & Joung, 2014). Genetically modified mice have been produced by injecting mRNA encoding the endonuclease Cas9, along with guide RNA (gRNA) targeting the gene to be modified, into mouse embryos (Wang et al., 2013). The CRISPR system can also be used to correct disease-causing mutations. Cataract-free mice have been generated by injecting Cas9 mRNA and gRNA targeting the mutated *Crygc* gene locus into mouse zygotes followed by implantation in surrogate female mice (Wu et al., 2013). Also, mutated cystic fibrosis transmembrane conductor receptor (CFTR) from the intestinal stem cells of cystic fibrosis patients has been corrected by the CRISPR/Cas system using gRNA targeting the mutated *CFTR* locus along with a plasmid DNA encoding the correct *CFTR* sequence as a template for homology-directed repair (Schwank et al., 2013). These preliminary examples support CRISPR-mediated genetic modifications as novel strategies to treat genetic diseases.

Proper genetic modification by CRISPR requires at least two components to be introduced to the target cells: first, the gRNA specific to the genetic locus to be modified; and second, the Cas9 endonuclease, which is usually introduced as a plasmid vector or mRNA. If the cell to be modified contains a homozygous mutation of the gene, an additional plasmid encoding the correct version of the gene is necessary in order to facilitate homology-directed repair of the mutation. It is obvious that all these nucleic acids require an efficient method to enter the target cells. The examples of CRISPR-mediated genetic modifications mentioned above used either commercially available transfection reagent, viral vectors or direct injection of CRISPR components into the target cells, which all have inferior in vivo performances as compared to lipid-based delivery systems. It is clear that our understanding of CRISPR/Cas system is still at the early stage; however, numerous companies have begun aggressive programs to develop research tools for the CRISPR field (reviewed in Baker, 2014). The race for the first CRISPR-based therapeutic for genetic diseases are currently underway and much like the development of siRNA-based drugs 10 years ago, the development of an effective delivery system for CRISPR components will be the key to this race. Based on its proven track record in nucleic acid delivery, it is possible that lipid-based systems will be at the forefront of this CRISPR technology. It remains to be seen whether CRISPR technologies will enjoy as much success as siRNA has in the clinic and it is

certain that we will see many innovations in both formulation method and delivery material for CRISPR in the near future.

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CHAPTER FIVE

Composite Nanoparticles for Gene Delivery

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Contents

Abstract

Nanoparticle-mediated gene and siRNA delivery has been an appealing area to gene therapists when they attempt to treat the diseases by manipulating the genetic information in the target cells. However, the advances in materials science could not keep up with the demand for multifunctional nanomaterials to achieve desired delivery efficiency. Researchers have thus taken an alternative approach to incorporate various materials into single composite nanoparticle using different fabrication methods. This approach allows nanoparticles to possess defined nanostructures as well as multiple functionalities to overcome the critical extracellular and intracellular barriers to successful gene delivery. This chapter will highlight the advances of fabrication methods that have the most potential to translate nanoparticles from bench to bedside. Furthermore, a major class of composite nanoparticle–lipid-based composite nanoparticles will be classified based on the components and reviewed in details.

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1. NANOMEDICINE AND GENE THERAPY

Nanomedicine generally refers to the medical application of nanotechnology. It is an interdisciplinary field that exploits the distinguishing features of nanomaterials to fulfill the demanding needs of future research and clinical purposes. Due to the fact that the nanomaterials fall in the same size range as biological molecules and vesicles, researchers are seeking to integrate nanomaterials with biology to develop novel diagnostic devices, contrast agents, analytic tools, and drug delivery carriers.

Nanoparticle-based drug delivery systems are gradually shifting the paradigm of the traditional pharmaceutical industry through targeted delivery and releasing therapeutics to specific cells in order to minimize undesired adverse effects. Moreover, some highly potent drugs with low bioavailability due to pharmaceutically unfavorable physical or chemical properties can now be formulated into nanoparticles, manifesting their real therapeutic efficacy. One such example is nucleic acid-based therapeutics, which encompass a large class of highly potent drugs.

The development of recombinant DNA technology has provided a tool to manipulate DNA and RNA sequences at will. This has led to the emergence of *Gene Therapy*, a promising technology that treats inherited or acquired diseases by introducing exogenous genetic information into specific cells of the patients (Mulligan, 1993). Later, the discovery of RNA interference expanded the field of gene therapy by administrating regulatory RNA molecules, specifically and effectively silencing the targeted gene expression (Fire et al., 1998). Both strategies are appealing to researchers due to the simplicity of the drug development. As long as the therapeutic targets are identified, a new drug can be instantly generated with high specificity and potency based on the genetic code. This cost-effective drug development strategy circumvents the high-throughput screening process for the development of chemically based drugs, which is costly and time consuming.

As promising as it looks, the clinical translation of gene therapy has been successful only in limited indications using viral vectors due to the physicochemical properties of nucleic acid drugs, such as vulnerability to nuclease degradation, high molecular weight, and anionic charge. All of these significantly reduce the bioavailability of the drugs after systemic administration. Therefore, advances in gene therapy demand the development of carriers to deliver the therapeutics to the target cells with high efficiency. Among all the viral or nonviral approaches, the development of nanoparticle-mediated gene delivery has been put on center stage due to the progress of materials science.

2. COMPOSITE NANOPARTICLES

The emergence of novel nanomaterials with outstanding physicochemical properties and biological performances has fueled the application of nanotechnology in gene delivery. The carriers fabricated with these materials have to go through a variety of physiological conditions, such as pHs, ionic and osmotic strengths after systemic administration. Meanwhile, the carriers need to keep their integrity during blood circulation and respond to particular stimuli for intracellular cargo release. However, it is difficult for single component-based nanoparticles to satisfy the complicated needs to achieve a sophisticated controlled-release platform for gene delivery.

Instead of developing a single novel material, it may be advantageous to fabricate carriers with multiple materials that are equipped with diverse functionalities. These classes of nanoparticles are referred to as *composite nanoparticles*. The fabrication of composite nanoparticles is an area of materials science, which has gained an increasing attention due to its scientific and technological importance. The most important step in developing these nanoparticles is the preparation of tailored composite nanostructures. To achieve structurally defined composite nanoparticles, virtually all physicochemical properties of the novel materials have been exploited to set up reproducible and well-controlled fabrication protocols, each one with its specific advantages and shortcomings.

In this chapter, we will review the three well-established and popular fabrication methods and analyze their pros and cons. Later, we will review the most extensively studied lipid-based core–shell-structured composite nanoparticles reported in recent years and categorize these nanoparticles based on the materials used. The specific features of the nanostructures as well as the properties of the materials will be discussed in light of their contributions to gene delivery.

3. FABRICATION METHODS OF COMPOSITE NANOPARTICLES

Composite nanoparticles are fabricated with various methods and usually with multiple steps due to the complicated composition of the nanoparticles. The synthesis methods of inorganic nanoparticles such as gold nanoparticles (Bao, Mitragotri, & Tong, 2013), magnetic nanoparticles (Cohen & Shoushan, 2013), quantum dots (Probst, Zrazhevskiy,

Bagalkot, & Gao, 2013), and silica nanoparticles (Fine et al., 2013), as well as polymeric nanoparticles (Feng et al., 2013) for drug delivery have been extensively reviewed. The composite nanoparticles, however, require further steps to incorporate other materials into the nanosystem. The fabrication of such nanoparticles primarily involves bulk mixing, which takes advantage of the physicochemical properties of the nanomaterials to achieve a defined nanostructure via self-assembly mechanism. As is recognized by the field of nanomedicine, bulk mixing often results in nanoparticles with large polydispersity as well as batch-to-batch variation. These issues represent critical challenges to clinical translation of the nanoparticles. To date, the applications of customized mixing devices and lithography technology to microfabrication have shown great potentials in solving the issues. Therefore, we will review the advances of the technologies in the fabrication of composite nanoparticles.

3.1 Self-assembly: Microfluidic Mixing

One of the major hurdles to the clinical translation of nanomedicine is the difficulty in reproducing batches of nanoparticles with identical properties in large-scale manufacturing for clinical use (Murday, Siegel, Stein, & Wright, 2009). Microfluidics is an interdisciplinary technology, which incorporates engineering, physics, chemistry, nanotechnology, and biotechnology, with extensive applications to systems in which small volumes of fluids are handled (10^{-9}–10^{-18} l). Microfluidics has expanded from chemical separations and its original semiconductor technology to the processing of ultralow sample volumes as well as accessing biological length scales. This expansion is also supported by the development of soft lithography, which allows rapid prototyping of microfluidic devices (McDonald et al., 2000), as well as the development of a simple method for fabricating pneumatically activated valves, mixers, and pumps (Thorsen, Maerkl, & Quake, 2002). These innovations significantly shorten the time needed to fabricate prototype devices for testing new ideas (Whitesides, 2006). In the drug delivery field, the applications of microfluidics are focused on the synthesis of nanoparticles. So far, this technology is anticipated to be the very solution to the reproducibility and large-scale issues for clinical evaluation (Valencia, Farokhzad, Karnik, & Langer, 2012).

Amphiphilic molecules such as lipids and copolymers will self-assemble into aggregates when the polarity of the solvent changes. The conventional way to cause this solvent change is to mix the molecularly favorable solvent with an unfavorable solvent. This forces the molecules to form nanoparticles. The mixing timescale (τ_{mix}) is usually a few

seconds, which is longer than the characteristic 10–100 ms timescale (τ_{agg}) for chains to aggregate (Valencia et al., 2012). The long mixing time causes the aggregates to be exposed to the heterogeneous solvent environment, preventing the effective stabilization of the nanoparticles by the hydrophilic portion of the molecules. This will lead to further aggregation of the molecules and result in larger, polydisperse nanoparticles. Microfluidics technology shortens the τ_{mix} from milliseconds to microseconds (Johnson & Prud'homme, 2003; Karnik et al., 2008) by mixing the two solvents in an ultrasmall volume. When $\tau_{mix} < \tau_{agg}$, the molecular aggregates are exposed to the homogenous solvent and the hydrophilic portion will stabilize the nanoparticle more effectively. In this way, smaller and more homogenous nanoparticles are produced.

Compared with macroscale mixing, which is achieved by a turbulent flow, the microfluidic system does not generate turbulence due to the hydrodynamic stability. In order to overcome this issue, microfluidic devices are designed to dramatically increase the effect of diffusion and advection by exploiting the small length of the system (Capretto, Cheng, Hill, & Zhang, 2011). Microfluidic mixing devices are generally classified as either passive or active mixing according to the design principle. Active micromixers use external energy input such as a pressure field, acoustics, or temperature to introduce perturbations, which result in efficient mixing. However, these devices require the integration of an energy source referred to as an actuator and is inconvenient to researchers working on chemical and biological applications. On the other hand, passive mixing relies entirely on pumping energy. The devices restructure the flow via channel design to maximize the contact surface area between flows. These devices are relatively inexpensive, convenient, and popular in nanoparticle engineering. Liu et al. (2010) have reported a digital droplet generator for the fabrication of nanoparticles with multiple building blocks including 1-adamantanamine (Ad)–polyamidoamine dendrimer conjugate, Ad-PEG (polyethylene glycol) conjugate, β-cyclodextrin–polyethylenimine (PEI) conjugate, and Ad-Arg-Gly-Asp (RGD)-PEG conjugate. The building blocks are introduced into the mixing device in a sequential pattern with digitally controlled processing parameters on a single chip. They have demonstrated successful fabrication of uniform, RGD-functionalized nanoparticles with defined sizes ranging from 30 to 350 nm. In addition, they are also capable of adjusting the density of targeting ligands on the nanoparticles (0–10% based on feeding) and correlating this with the cellular uptake efficiency (Liu et al., 2010). Rhee et al. have demonstrated preparation of (polylactic-co-glycolic acid)

PLGA-PEG nanoparticles using a simplified 3D hydrodynamic focusing technique in microfluidic channel design (Figure 5.1) (Rhee et al., 2011). This device is composed of a monolithic single layer with three sequential vertical inlets followed by horizontal focusing. This method avoids the clogging of channels due to the aggregation of high molecular weight polymers in the channel walls. Valencia et al. (2010) have demonstrated the fabrication of a core–shell-structured nanoparticle composed of PLGA in the core, lipid in the core–shell interface, and PEG on the surface (Figure 5.1 B, C and D). Their microfluidic device has passive mixing Tesla structures built into the mixing channels to facilitate the formation

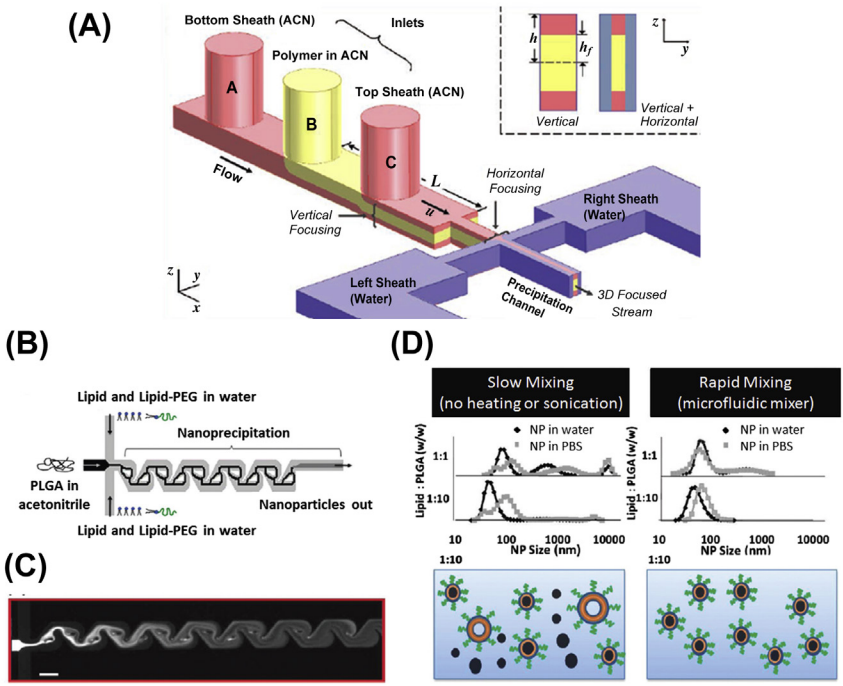

Figure 5.1 (A) Schematic illustration of 3D hydrodynamic focusing composed of three inlets for vertical focusing and separate inlet for side sheath flows. *(Reprinted with permission from Rhee et al. (2011).)* (B) Schematic illustration of the microfluidic device with three inlets that allows the formation of lipid-coated polymeric nanoparticles in the microchannels with Tesla structures. (C) Solvent mixing in the Tesla channels using fluorescent dye and water respectively. The mixing is complete at the fourth turn in the channel. (D) Comparison of slow and rapid mixing of lipid and PLGA solutions. Aggregation forms under slow mixing conditions without the input of any energy, but not under rapid mixing conditions. (See the color plate.) *(Reprinted with permission from Valencia et al. (2010).)*

of nanoprecipitation. They have shown that a single, rapid mixing of PLGA in acetonitrile and lipid/lipid-PEG micelles in water using hydrodynamic flow focusing can fabricate homogenous nanoparticles with narrow size distribution. Similarly, they were able to control the physicochemical properties of the nanoparticles such as zeta potential, size, and surface functionalization. It is also noteworthy that the nanoparticles fabricated in one-step mixing showed no difference from the nanoparticles formed by mixing lipid with preformed PLGA cores. The explanation by the author was that the formation of the PLGA core was not affected by the presence of lipids. It is unclear if this one-step mixing could be applied to core–shell-structured nanoparticles made of other materials (Valencia et al., 2010).

Although nanoparticles prepared with microfluidic devices are often in micro- to milligram scale, the stackability and reproducibility of this preparation allows gram to kilogram scale manufacturing of nanoparticles for clinical evaluation. This undoubtedly is the most feasible approach to commercialize self-assembled nanomedicine to comply with good manufacturing practices.

3.2 Self-assembly: Layer-by-Layer

Layer-by-layer (LbL) self-assembly is a method that can be used to construct nanoparticles with multilayer structures. This method involves alternative and repetitive adsorption of materials with opposite charges on the surface of the core materials (Deshmukh et al., 2013). In 2001, Qiu et al. applied the LbL method to the fabrication of ibuprofen microparticles. Biocompatible polyelectrolytes such as chitosan, dextran sulfate, carboxymethyl cellulose, and alginate were used as coating materials to fabricate polyelectrolyte microcapsules with a shell as thick as 20–60 nm. The capsule thickness affects the release rate of the drugs. This represented the first LbL-based self-assembly system in drug delivery (Qiu, Leporatti, Donath, & Mohwald, 2001). Electrostatic interaction between oppositely charged polyelectrolytes is considered to be the major stabilizing force. However, hydrogen bonding, hydrophobic interactions, and van der Waals forces contribute to LbL formation as well (de Villiers, Otto, Strydom, & Lvov, 2011). The coating stability, morphology, thickness, drug depositions, and permeation of the film are primarily affected by these forces (Hammond, 1999; Lvov, Ariga, Onda, Ichinose, & Kunitake, 1999).

LbL-based multilayers offer several distinct advantages compared to other fabrication methods. (1) The thickness of the walls can be tailored to

control the particle size; (2) The selection of polyelectrolytes for coating can be engineered to control the stability of the nanoparticle; (3) the location and sequence of the layers can be controlled to manipulate the drug release kinetics.

3.3 Imprint Lithography: PRINT (Particle Replication in Nonwetting Templates) Technology

The imprint lithography-based method is considered to be a promising technique for scalable preparation of colloidal particles with specific shape and size (Merkel et al., 2010). This technology involves the use of a rigid template for casting an elastomeric mold, which will be used to replicate the shape of the original template (Qin, Xia, & Whitesides, 2010). It features high resolution, high fidelity, and low cost for large-scale manufacturing of particles. PRINT is a top-down fabrication method that is capable of producing uniform, micro- and nanoparticles with absolute control over size, shape, and composition. This versatile technology can be applied to fabricate particles with a variety of chemical structures. In 2004, DeSimone et al. reported the successfully generation of nanoparticles using photocurable perfluoropolyether (PFPE)-based materials with high-resolution imprint lithography (Figure 5.2) (Rolland, Hagberg, Denison, Carter, & De Simone, 2004). They have developed a chemically robust and durable PFPE-based mold that is also solvent resistant. More importantly, PFPE solved the swelling (Lee, Park, & Whitesides, 2003) and surface energy issues of polydimethylsiloxane-based soft lithography and allowed

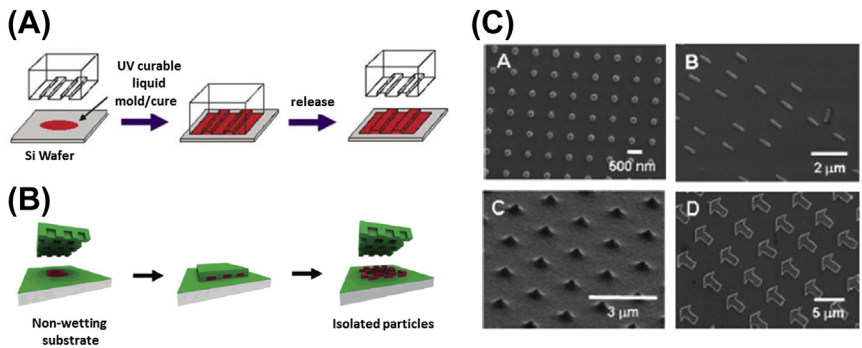

Figure 5.2 (A) Schematic illustration of the imprint lithography process. *(Reprinted with permission from Rolland et al. (2004).)* (B) Schematic illustration of the PRINT process. In PRINT, the nonwetting feature allows the generation of isolated particles. (C) Manipulation of PRINT nanoparticles with different shape and size. (See the color plate.) *(Reprinted with permission from Rolland et al. (2005).)*

the fabrication of nanomaterials with high fidelity and quality (Rolland et al., 2004). Another impressive feature of PRINT compared with traditional imprint lithography is that PFPE-based molds are nonwetting to both inorganic and organic materials (Rolland, Van Dam, Schorzman, Quake, & DeSimone, 2004). This unique feature allows the production of isolated nanoparticles instead of embossed films. With this PFPE-based mold, they were able to fabricate monodispersed particles with a variety of materials including PEG, poly-(D-lactic acid), poly-(pyrrole), and triacrylate resin. Also, they were able to incorporate fragile biological molecules such as DNA or protein into these particles without sacrificing their activity (Rolland et al., 2005). Later, Kelly et al. applied this technology to protein-based materials. They molded insulin, albumin, and albumin mixtures with siRNA or paclitaxel using PRINT technology and generated uniform nano- and microparticles that have potential applications in drug delivery (Kelly & DeSimone, 2008).

The unique feature of imprint lithography-based fabrication compared to self-assembled nanoparticles is the ability to control the shape of the particles. Therefore a variety of shapes including cylinders, spheres, prolate ellipsoids, and toroidal particles can be fabricated using this technology. As potential drug delivery carriers, the cellular uptake efficiency is usually affected by size, surface charge, targeting ligands, and shape. The first three parameters can be manipulated by adjusting the input of building blocks during fabrication. However, shape can only be controlled by using a mold. Therefore, imprint lithography offers a useful tool to study the correlation between the particle shape and cellular uptake efficiency. It was demonstrated that HeLa cells can readily internalize both cubic and cylindrical particles with dimensions as large as 3 μm. However, the cylindrical particles were more efficiently taken up. A higher aspect ratio also contributes to the uptake rate (Gratton et al., 2008). Therefore, the shape of the particle is taken into consideration for rational design of drug delivery carriers.

4. COMPOSITE NANOPARTICLES FOR TARGETED GENE DELIVERY

The pursuit of successful delivery of targeted nucleic acid-based therapeutics will never cease. Numerous talented researchers are committed to the engineering and development of ideal nanoparticles to achieve this goal. With their efforts, various sophisticated nanoparticle platforms have been devised that have demonstrated high delivery efficiency and specificity. Material-oriented innovation has been the driving force of the field. However, researchers have

started to realize that it is highly unlikely that one material is capable of dealing with all the barriers present in gene delivery. Failure to overcome anyone of the barriers will significantly compromise the delivery efficiency. In order to resolve this problem, distinct functional materials are chosen and incorporated into one nanoparticle to overcome these barriers. This modular-based design approach has turned out to be very effective in engineering nanoparticles for efficient gene delivery. There are a large number of publications on these composite nanoparticles and their sophisticated design to overcome the critical barriers to gene delivery. The barriers to gene delivery are: (1) The adsorption of serum protein compromises the colloidal stability of nanoparticles after systemic administration; (2) the reticuloendothelial system takes up the nanoparticles in the blood circulation; (3) the cytoplasmic membrane prevents the nanoparticles from entering the cells; (4) the nanoparticles are trapped in the endo/lysosome compartment; (5) the nuclear envelope blocks the DNA from entering the nucleus for transcription. We will review the recent advances of composite nanoparticles in this section.

4.1 Lipid-Coated Composite Nanoparticles

Lipids have been extensively used in the fabrication of composite nanoparticles because they can be easily formulated into other nanoformulations through electrostatic or hydrophobic interaction. Lipid coating allows the nanoparticles to acquire liposomal characteristics. The surface of the lipid-coated nanoparticles can be easily manipulated with simple and well-established protocols for liposome modification. For example, PEGylation of nanoparticles can be achieved by postinsertion of lipid-PEG conjugates. Targeting ligands or other functional motifs can be incorporated in the same way. These nanoparticles are PEGylated with lipid-PEG conjugates by simultaneous or postinsertion, giving the nanoparticles a stealth property after systemic administration. The cores of these lipid-coated composite nanoparticles are diverse and can be classified into several categories, which include self-assembled cores such as polyplexes or polymeric nanoparticles, as well as inorganic cores such as gold, silica nanoparticle, or calcium phosphate nanoparticles (details Haynes et al., this book).

4.1.1 Polyplex-Based Cores

Polyplexes refer to nanosized complexes between negatively charged polyanions such as nucleic acids and positively charged polycations including synthetic polymers (polyethyleneimine, polylysine), polypeptides (protamine, spermidine, or histone), and natural polymers (chitosan). These nanoparticles are widely used as gene delivery carriers for siRNA and DNA in vitro.

However, due to serum instability and nonspecific uptake by reticuloendothelial system (RES) after systemic administration, their applications in vivo are limited. One of the most exploited approaches to address this issue is coating the polyplexes with lipids. A lipid bilayer spontaneously forms on the surface of the polyplexes when mixing the polyplexes with preformed liposomes or rehydrating the dry lipid membrane with the polyplex solution. This approach takes advantage of the low toxicity and immunogenicity of liposomes along with their ability to be PEGylated for extended circulation.

Most representative examples of these core-membrane nanoparticles have been developed in Dr Leaf Huang's lab. As early as the mid-1990s, Li et al. formulated these core-membrane nanoparticles using poly(L-lysine) and protamine (Li & Huang, 1997) to condense pDNA into negatively charged polyplexes. The cores were then coated with a preformed cationic liposome containing 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and dioleoyl-phosphatidylethanolamine (DOPE) for lipid coating. These core-membrane-structured composite nanoparticles were named lipid–protamine–DNA (LPD) nanoparticles. Compared with lipoplexes formed by cationic liposomes and pDNA, the size of LPD nanoparticles dramatically decreased and effectively protected DNA from nuclease degradation (Gao & Huang, 1996). This lipid coating enhanced the transfection efficiency of the lipid nanoparticles by up to 28-fold in vitro compared with lipoplexes (Gao & Huang, 1996). LPD was also able to transfect the lungs in vivo with minor modifications; possibly due to the excessively positive charge carried by the nanoparticles (Li & Huang, 1997). Later, Li et al. optimized LPD nanoparticles by incubating the lipid-coated LPD with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)] (DSPE-PEG) micelles to PEGylate the nanoparticle. PEGylation reduced the serum protein adsorption and increased the colloidal stability of the nanoparticles. Therefore, less nanoparticles were taken up by the RES system and a higher tumor accumulation was achieved due to the enhanced permeation and retention effect (Li, Chen, Hackett, & Huang, 2008; Li, Chono, & Huang, 2008). This PEGylated LPD substantially increased in vivo siRNA delivery efficiency by fourfold and the gene silencing effect by two- to threefold (Li & Huang, 2006). It is worth noting that the negatively charged protamine/calf thymus DNA/siRNA core played a critical role in supporting the lipid bilayer, allowing more surfactant-like DSPE-PEG conjugates to be inserted on the membrane without detaching the lipid membrane (Li & Huang, 2009). This high density of sheddable PEG significantly improved the PK of LPD nanoparticles in the blood stream (Li & Huang, 2010). An outstanding issue that remained

for LPD nanoparticles was the high binding affinity between nucleic acid and protamine, which was so strong that cytoplasmic release of the cargos became insufficient. This could compromise delivery efficiency as well. To address this issue, Wang et al. engineered a histone-derived fusion protein to replace protamine for nucleic acid condensation. The fusion protein is composed of four repeats of histone H2 peptide derived from *N*-terminal of histone H2 protein as the condensing element. The tandem repeats were linked with the enzyme-responsive degradation element, cathepsin D cleavage substrate. The exposure of the fusion protein to the corresponding enzyme in the endosome led to the degradation of the fusion protein. This degradation mechanism engineered in the condensation agent facilitated the cargos release and efficiently increased the in vivo target gene knock down efficiency by twofold (Wang, Zhang, Guo, Hatefi, & Huang, 2013) (Figure 5.3).

A similar approach has been employed by Harashima et al., who have developed a series of lipid-based core-membrane-structured nanoparticles, namely multifunctional envelope-type nanodevice (MEND) (Akita et al., 2009; El-Sayed, Masuda, Khalil, Akita, & Harashima, 2009; Hatakeyama, Akita, & Harashima, 2011; Hatakeyama, Akita, Ito, et al., 2011; Hatakeyama et al., 2007, 2009; Ishitsuka, Akita, & Harashima, 2011; Khalil, Hayashi, Mizuno, & Harashima, 2011; Khalil et al., 2007; Kogure et al., 2004; Kuramoto et al., 2008; Masuda et al., 2008; Moriguchi et al., 2005; Mudhakir, Akita, Tan, & Harashima, 2008; Nakamura, Kogure, Futaki, & Harashima, 2007; Nakamura, Kogure, Yamada, Futaki, & Harashima, 2006; Sasaki et al., 2005; Shaheen et al., 2011). However, the lipid-coating procedure for MEND nanoparticles is slightly different from LPD nanoparticles. Instead of using preformed liposomes, the polyplex solution is used to rehydrate a lipid film, which had been dried down from an organic solvent solution. The hydration initiates the formation of the liposome, which subsequently coats the polyplex condensates. In those formulations, stearyl-octaarginine and cholesteryl-GALA (WEAALAEALAEALAEHLAEALAEALEALAA) are incorporated into the membrane for efficient cellular uptake and endosomal escape as a pH responsive fusogenic peptide (Hatakeyama et al., 2009; Khalil et al., 2007, 2011; Nakamura et al., 2006, 2007). The final nanoparticle is sonicated for self-assembly and homogeneity. MEND has been employed to deliver genes to the liver (Khalil et al., 2011) or the lung (Ishitsuka et al., 2011) with surface modifications of octaarginine or IRQ peptide (IRQRRRR), respectively. The incorporation of octaarginine and IRQ peptide facilitate the internalization of MEND nanoparticles through macropinocytosis or caveolar endocytosis, respectively.

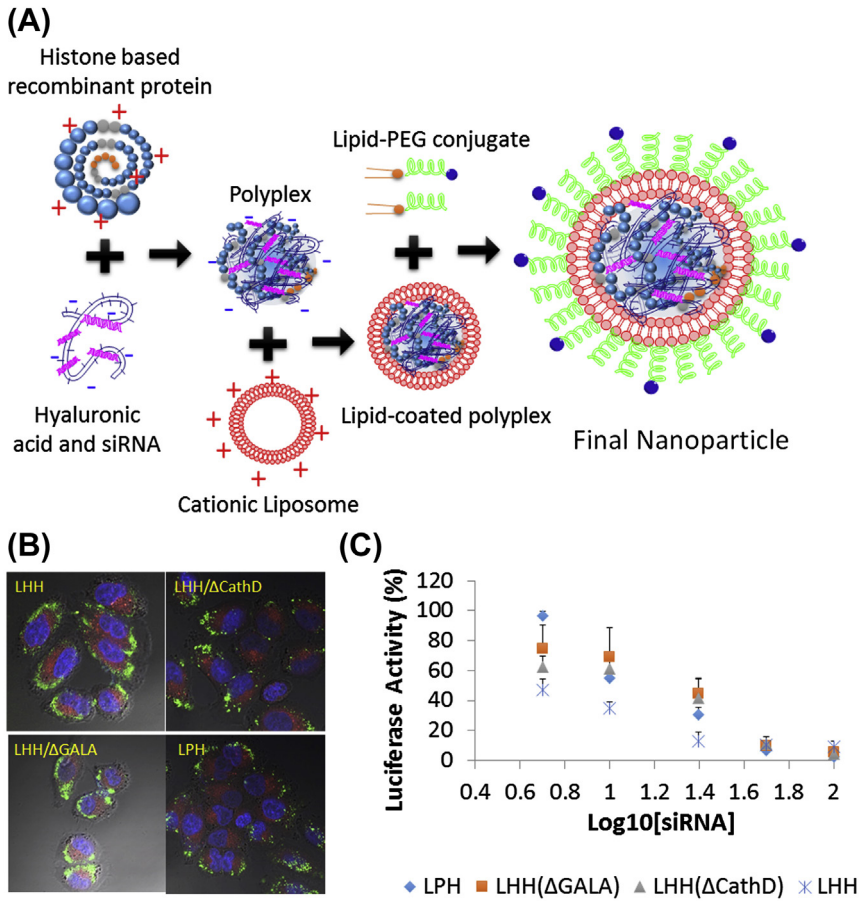

Figure 5.3 (A) Schematic illustration of fabrication of polyplex core-based composite nanoparticle for siRNA delivery using histone-based recombinant protein as nucleic acid condensing agent. (B) Intracellular release profiles of oligonucleotides using the different histone-based recombinant proteins and mutants. Lack of degradation element (LHH/ΔCathD) prohibited the dissociation of oligonucleotides and the punctate forms were observed. (C) IC50 of luciferase knockdown with antiluciferase siRNA delivered by different nanoparticles. (See the color plate.) *Reprinted with permission from Wang et al. (2013).*

4.1.2 Mesoporous Silica Nanoparticle Cores

Mesoporous silica nanoparticles (MSNs) are one of the most well-studied inorganic nanoparticles for the delivery of drugs and contrast agents. These nanoparticles possess some unique characteristics such as high drug-loading capacity due to large surface area and high pore volume as well as tunable pore and particle size. The surfaces of MSNs are also subject to a variety of modifications. In order to deliver nucleic acid agents, the silica surface of the MSNs is

converted to carry positive charges for the binding of DNA or siRNA. This is realized by grafting an amine group on the surface (Kneuer et al., 2000) or coating the MSNs with polycations such as PEI (Meng et al., 2010; Shen et al., 2014) and polyamidoamine (Radu et al., 2004). Due to the relatively small pore sizes of MSNs, nucleic acid therapeutics such as DNA and siRNA are usually immobilized on the external surface of the MSNs. Recent advances have engineered MSNs with large pores, which make encapsulation of genes possible. Min et al. synthesized 250 nm monodispersed MSNs with large cationic pores (>15 nm) to load plasmid DNA (Kim et al., 2011). This strategy held DNA inside and provided better protection against nuclease degradation. Qiao et al. fabricated 100–200 nm MSNs that contain 28 nm cage-like pores and a large entry size of 13.4 nm. This is large enough to encapsulate siRNA with a polylysine surface functionalization (Hartono et al., 2012).

The Brinker lab is a pioneer for the engineering of MSN-based composite nanoparticles, referred to as "protocells" (Liu, Stace-Naughton, Jiang, & Brinker, 2009). The protocells are fabricated by fusing liposomes on MSNs and simultaneously loading and sealing the cargos inside the particles with the lipid membrane. Compared to the traditional liposomes, protocells have demonstrated higher drug-loading capacity and stability (Ashley et al., 2011). They are able to encapsulate a variety of cargos including chemodrugs, proteins, quantum dots, and siRNAs (Ashley et al., 2011). Ashley et al. exploited the emulsion processing technique (Carroll, Pylypenko, Atanassov, & Petsev, 2009) to prepare 165 nm MSNs with ultralarge pores (23–30 nm) (Ashley et al., 2012). These MSNs were characterized by a Brunauer–Emmett–Teller surface area of 850 m^2/g and a pore volume fraction of 65% with 3–13 interconnecting surface accessible pores. Surface modification of MSNs with 3-[2-(2-aminoethylamino)ethylamino]propyltrimethyoxysilane resulted in a siRNA-loading capacity of about 1 nmol per 10^{10} particles. Cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)- or zwitterionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)-based lipid coating did not cause much of a difference in siRNA loading, although both types of lipid-coated MSNs showed 10–100 times higher loading than the corresponding lipid nanoparticles. These results confirmed that high surface area cores confer a higher intrinsic loading capacity. To facilitate the targeted cellular uptake and endosomal escape of the cargos, the protocells were surface modified with targeting peptide and lipidated endosomolytic peptide (H5WYG) using the postinsertion method. In vitro studies have shown that the delivery of siRNA using lipid-coated MSNs is able to silence the expression of the target protein by 90% in 72 h (Ashley et al.,

2012). In the same group, Dengler et al. reported the delivery of pDNA using a similar platform. The MSNs in this study were prepared by the surfactant template aerosol-assisted self-assembly method (Liu et al., 2009) and surface modified with ATPES to carry a positive charge. These 230 nm MSNs are characterized by a surface area of 935 m^2/g and a small pore size of 2–5 nm. During the pDNA loading procedure, the positively charged MSNs adsorbed the pDNA to the external surface of the nanoparticles and was then followed by lipid coating. Due to the extralarge size of the pDNA, the cargo was not meant to be loaded inside the MSNs. Hypothetically, the pDNA were trapped between the nanoparticle surface and lipid layer, although this was not confirmed experimentally. Without a targeting ligand, DOTAP coating of the MSNs resulted in a much higher transgene expression level (Interleukin 10) than DOPC-coated MSNs in HEK cells. Presumably, this is due to enhanced cellular uptake and endosomal escape caused by the cationic lipid. Although the composite MSNs particles have shown capacity for siRNA and pDNA delivery, the loading mechanism is better characterized for siRNA. In contrast, the loading of pDNA primarily depends on surface adsorption to the positively charged nanoparticles, which provides negative charges for the cationic liposomes to bind and fuse. However, whether the cationic liposomes have a chance to compete and rip off the pDNA from the surface of the MSNs, is not well investigated. With advances in MSN fabrication technology, the ultralarge pore size could be engineered to accommodate the loading of pDNA inside the particles.

4.1.3 Polymeric Nanoparticle-Based Core

Biodegradable and biocompatible polymers are a dominant class of nanomaterials for drug delivery. This can be seen through an increasing number of publications and clinical trials. Polymeric nanoparticles (PNPs) exhibit high structural integrity, stability, and controlled release. They are fabricated with distinct synthetic polymers or modified natural polymers through a variety of preparation methods (Hadinoto, Sundaresan, & Cheow, 2013). Therefore rational design of the building block polymers allows fine-tuning of the size, charge, shape, and functionality of the nanoparticles (Panyam & Labhasetwar, 2003). For the aforementioned reasons, lipid-coated polymeric nanoparticles have been developed in an effort to mitigate the shortcomings of PNPs such as in vivo instability, nonspecific toxicity, and early drug release. The lipid-coated PNPs are engineered to load chemodrugs, nucleic acid therapeutics, proteins and peptides by entrapment, adsorption or covalent conjugation. The nanoparticles have a well-defined core–shell

nanostructure, which is composed of three parts: (1) the polymeric core where the drugs are loaded; (2) the lipid membrane, which confers the liposomal characteristics to the core and prevents the leakage of the drugs from the core; and (3) the PEG layer, which can also be functionalized for targeting, membrane penetration, or endosomal escape. The most commonly studied polymers are PLGA and polycarprolactone due to their low cost of synthesis and easy functionalization.

In order to load nucleic acids into PLGA nanoparticles, a polycation-based condensing agent is usually used to condense and increase the hydrophobicity of the cargos so that they can be loaded into the hydrophobic core. Zhong et al. (2010) have characterized three methods to encapsulate pDNA in a lipid-PLGA-based composite nanoparticle formulation prepared with a conventional double emulsion method (W/O/W). These pDNA loading methods were designated as: (1) "OUT," pDNA is adsorbed at the surface of the nanoparticles; (2) "IN," pDNA is encapsulated in the PLGA core of the nanoparticles; (3) "BOTH," pDNA is adsorbed and encapsulated in the nanoparticles. This was realized by adding protamine-condensed pDNA before and/or after the formation of PLGA core. In vitro release and transfection studies using luciferase reporter gene in human embryonic kidney cells indicated that the "OUT" loading method tended to cause a burst effect of transgene expression with shorter expression longevity. "IN" and "BOTH" loading methods could generate a sustained expression profile, which indicated a slow release of the cargo due to the hydrolysis of PLGA (Zhong et al., 2010).

Numerous formulations have been developed for the delivery of siRNA using lipid/polymer composite nanoparticles (Desai et al., 2013; Gao et al., 2014; Shi, Xiao, Votruba, Vilos, & Farokhzad, 2011; Yang et al., 2012) (Figure 5.4). Desai et al. (2013) exploited the OUT loading method to codeliver anti-TNFα siRNA and antinociception agent Capsaicin with lipid-coated PLGA nanoparticles prepared through the one-step double emulsion method. Capsaicin was encapsulated into the core and siRNA was adsorbed on the surface of cationic lipid membrane. The topical administration of the nanoparticles significantly reduced inflammatory cytokines, such as TNF-α, NK-κB, and IL-17 and effectively treated chronic skin inflammation on imiquimod-induced psoriatic plague-like model (Desai et al., 2013). Shi et al. (2011) have reported hollow-structured lipid-PLGA composite nanoparticles that encapsulated siRNA in the cavity of the nanoparticle. The nanoparticle was formulated by adding a siRNA solution to an organic solvent in the presence of ethylphosphocholine (EPC) and PLGA. The amphiphilic EPC stabilized the water-in-oil microemulsion with the

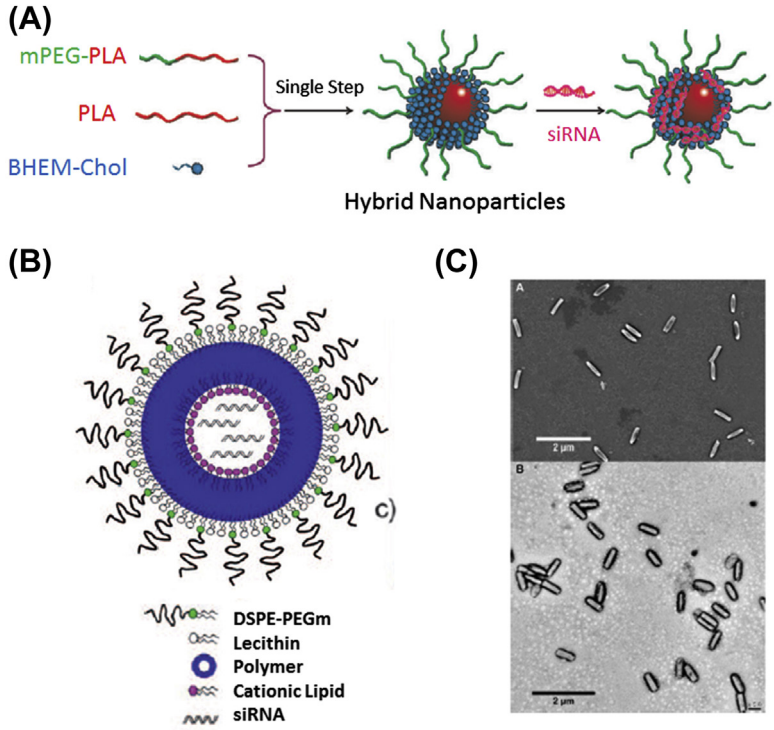

Figure 5.4 (A) Schematic illustration of fabrication of lipid-coated Polylactic acid (PLA) nanoparticles with siRNA adsorbed on the surface of carrier. *(Reprinted with permission from Yang et al. (2012).)* (B) Schematic representation of lipid–polymer nanoparticle composed of outer lipid-PEG, a middle polymer layer, and an inner cationic lipid hollow core for siRNA entrapment. *(Reprinted with permission from Shi et al. (2011).)* (C) SEM (top) and TEM (bottom) images of lipid-coated PRINT nanoparticles composed of PLGA and siRNA. (See the color plate.) *(Reprinted with permission from Hasan et al. (2012).)*

hydrophilic head in the aqueous phase and hydrophobic chain in the PLGA phase. This allows the effective encapsulation of siRNA in the aqueous phase. In a second emulsion and subsequent solvent evaporation, DSPE-PEG coated on the surface of PLGA and formed an outer lipid-PEG layer. The lipid bilayer was separated by ester-terminated PLGA and the hollow structure densely loaded siRNA for delivery (Shi et al., 2011).

Gao et al. reported a composite nanoparticle platform composed of a cholesterol-grafted poly(amidoamine) (rPAA-Chol) core and a lipid coating (Gao et al., 2014). The amphiphilic polymer self-assembled into a nanoparticle in aqueous solution using the thin-film hydration method, followed by the addition of siRNA molecules. The anionic core was then

coated with DOTAP-based cationic liposomes for lipid coating. The lipid–polymer nanoparticles were then subject to PEGylation by postinsertion of a lipid-PEG conjugate. An endosomolytic peptide, T7 (HAIYPRH), or transferrin, was conjugated to the distal end of the PEG chain for either enhanced endosomal escape or targeting purposes. The lipid-rPAA-Chol composite nanoparticles were relatively small in size (100 nm) compared to lipid-PLGA nanoparticles and showed a significant tumor inhibition effect when delivering anti-EGFR siRNA to an MCF-7 bearing tumor model (Gao et al., 2014).

Recently, Hasan et al. reported the fabrication of lipid-PLGA nanoparticles using PRINT technology to generate a PLGA/siRNA core with high siRNA loading (Hasan et al., 2012). The siRNA and PLGA (85:15 lactic/glycolic acid) were prepared in Dimethyl sulfoxide/Dimethylformamide/water and molded using PRINT technology. The 80×320 nm monodispersed needle-shaped core was fabricated for better cellular uptake. The core was then subject to cationic lipid coating and PEGylation. These particles have been tested in multiple cell lines and target genes knockdown has been demonstrated. This study was a hallmark for the development of composite nanoparticle-based siRNA delivery. The nanoparticles showed great translational potential due to their high reproducibility from batch to batch and a relatively easy scale-up production for clinical evaluation (Hasan et al., 2012).

4.1.4 Gold Nanoparticle-Based Cores

The gold nanoparticles (AuNPs) draw much attention as their applications in bimolecular sensing, computed tomography imaging, as well as photothermal therapy. Over the past decades, AuNPs have also been investigated as efficient nucleic acid delivery systems (Ding et al., 2014) due to their biocompatibility, versatility, and facile surface modification through gold–thiol linkages.

Chad Mirkin and his colleagues have been dedicated to the investigation of such nucleic acid-gold nanostructures as well as their biomedical applications (Barnaby, Lee, & Mirkin, 2014; Choi, Hao, Narayan, Auyeung, & Mirkin, 2013; Giljohann, Seferos, Prigodich, Patel, & Mirkin, 2009; Jensen et al., 2013). In their studies, citrate-stabilized AuNPs were mixed with thiolated siRNA, where siRNAs duplexes were allowed to chemisorb via thio–gold bonds (Giljohann et al., 2009). The dense surface functionalization of nucleic acids increases the stability of the bound cargos, while maintaining the biological activity of the siRNAs. However, negatively charged, nucleic acid-adsorbed AuNPs showed

compromised cellular uptake due to the weak interactions between the cellular membrane and nanoparticles. To address this issue, Rhim, Kim, & Nam (2008) coated nucleic acid-adsorbed AuNPs with cationic liposomes to enhance the cellular uptake and protect cargos from nuclease-mediated degradation (Figure 5.5). In this study, they used AuNPs of various sizes (15, 30, 50, and 80 nm) to load pDNA for lipid coating. Two types of lipid–gold nanoparticles were observed under cryo-TEM observation. Type I hybrids were believed to be the intermediate status, which showed the adsorption of AuNPs to the surface of liposomes. Type II hybrids showed that AuNPs were encapsulated in the liposomes, and the diameters of the lipid membrane were much larger than those of AuNPs. The cryo-TEM images suggested that the lipid membrane was not supported by the AuNP core. Although the author did not give the explanation for the structural discrepancy between lipid-AuNPs and other lipid-coated nanoparticles, it could be speculated that the small AuNPs with high curvature made it difficult for the lipid to form bilayer on them. The in vitro transfection studies showed that lipid-coated AuNPs resulted in significantly higher transfection efficiency compared with liposome-mediated or naked AuNPs-mediated DNA delivery due to the high DNA loading capacity and higher cellular uptake. Recently, Kong et al. reported a distinct method for the fabrication of lipid-coated AuNP for siRNA delivery using 5 nm AuNPs. The AuNPs were dissolved in chloroform in the presence of DC-Chol, DOPE, and cholesterol. The system was then emulsified followed by the evaporation of the solvent. Final particles contained cationic lipid membrane with cluster of gold nanoparticle cores. siRNAs were adsorbed on the surface

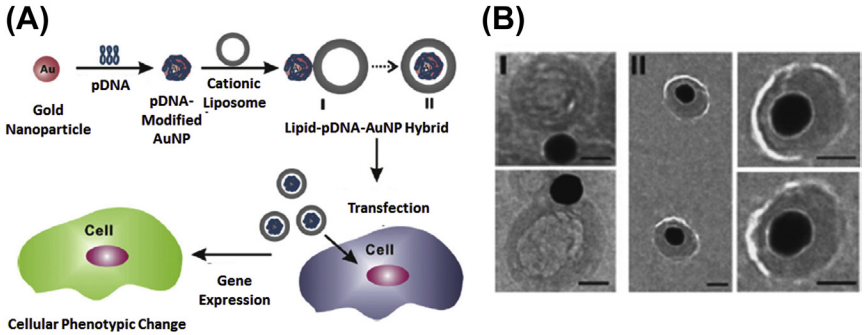

Figure 5.5 (A) Schematic illustration of lipid-coated AuNP for the delivery of plasmid DNA. (B) Cryo-TEM images of two types of lipid-coated AuNPs loaded with pDNA. Scale bars: 50 nm. *Reprinted with permission from Rhim et al. (2008).*

of such nanoparticles via electrostatic interaction (Kong, Bae, Jo, Kim, & Park, 2012). Recently, Alhasan et al. have introduced an approach to take advantage of cells for the fabrication of lipid–gold composite nanoparticle (Alhasan, Patel, Choi, & Mirkin, 2014). In their study, it was discovered that when endocytosed into PC-3 prostate cancer cells, a small percent of the nucleic acid-adsorbed gold nanoparticles could be naturally sorted into exosomes. These AuNPs-containing exosomes could be isolated and enriched for in vivo miRNA delivery. Although the coating and loading efficiency for the nanoparticles fabricated with this method was not efficient, the approach represented engineered nanoparticle of biogenesis, which is a trend regarding the fabrication of the nanoparticles.

4.1.5 Magnetic Nanoparticle-Based Core

Magnetic field has been exploited as an external energy to enhance the transfection efficiency of nonviral-mediated gene delivery (Duan et al., 2014; Hao et al., 2010; Miao et al., 2014; Scherer et al., 2002). For efficient in vivo delivery of siRNA, Namiki et al. have prepared lipid-coated magnetic nanocrystals termed "LipoMag." LipoMags were fabricated by dispersing oleic acid-coated magnetite nanocrystal cores with cationic lipid in chloroform and removing solvent afterward. Final nanoparticles were generated by spontaneously coating of monolayer cationic lipid on top of the nanocrystal cores via hydrophobic interactions. The siRNAs were then adsorbed on the surface of the LipoMag through electrostatic interaction. Under a magnetic field, heavy accumulation of siRNA delivered by LipoMag was observed in the tumor lesions compared with other organs, which resulted in target gene silencing (Namiki et al., 2009). Recently, Jiang, Eltoukhy, Love, Langer, and Anderson (2013) reported a simple method to coat iron oxide nanoparticles with lipid or lipid-like materials and form uniform nanoparticles. In their method, the solvent *N*-methyl-2-pyrrolidone (NMP) was added to the chloroform where oleic acid-coated iron oxide nanoparticles and lipids were dispersed. NMP serves as adhesive between the lipids and nanoparticle surface, which could be removed by dialyzing nanoparticles against water since it is miscible with both chloroform and water. Introduction of NMP promoted the full arrangement and assembly of outer leaflet lipid into a more complete layer and prevented the aggregation from happening. The nanoparticles generated were highly homogenous and showed high efficiency in delivering pDNA and siRNA in vitro (Jiang et al., 2013).

5. CONCLUSION

The chapter highlights core–shell-structured composite nanoparticles for gene therapy with a focus on lipid-coated systems. The fusion of lipid with other nanomaterials renders colloidal stability as well as other liposome-like properties to the composite nanoparticles, which makes them suitable carriers for in vitro and in vivo delivery. In addition, the well-defined nanostructure allows modular design of multifunctional nanoparticle, with each component overcoming designated extracellular or intracellular barriers to efficient gene delivery in a spatiotemporal manner. The fabrication of composite nanoparticles lowers the demand for the development of omnipotent nanomaterials for nonviral gene delivery and accelerates the advances in clinical translation of the nanomedicine. Moreover, some inorganic components such as gold or magnetic could be used for thermal activation and as contrast agent for noninvasive diagnosis of the cancers as well as monitoring of drug distribution. Therefore, lipid-based composite nanoparticle clearly affords new options for gene therapy and hold great potential for clinical translation.

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CHAPTER SIX

Multifunctional Enveloped Nanodevices (MENDs)

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Contents

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Abstract

It is anticipated that nucleic acid medicines will be in widespread use in the future, since they have the potential to cure diseases based on molecular mechanisms at the level of gene expression. However, intelligent delivery systems are required to achieve nucleic acid therapy, since they can perform their function only when they reach the intracellular site of action. We have been developing a multifunctional envelope-type nanodevice abbreviated as MEND, which consists of functional nucleic acids as a core and lipid envelope, and can control not only biodistribution but also the intracellular trafficking of nucleic acids. In this chapter, we review the development and evolution of the MEND by providing several successful examples, including the R8-MEND, the KALA-MEND, the MITO-Porter, the YSK-MEND, and the PALM.

1. INTRODUCTION

In the twenty-first century, a paradigm-shift occurred in drug discovery and development with the emergence of new drugs derived from small molecular compounds (van der Greef & McBurney, 2005). The expiration of patents for blockbusters such as Atorvastatin (Lipitor) is referred to as a Patent Clift. Many efforts have been expended by Big Pharmas to develop new drugs derived from small molecular weight compounds. The development

and use of antibodies followed small molecular compounds as new drugs. Although the monoclonal antibody was developed in the 1970s, it was only developed as a medicine after 30 years. The use of antibodies expanded fields of therapy such as cancer and immunodeficiencies which cannot be treated by low molecular weight compounds. More than half of the top 10 drugs on the market are antibody-based drugs and many of these are in the clinical trial stage. An antibody which can recognize a specific target molecule compared to other types of drugs (Yao, Zhu, & Chen, 2013), however, they cannot target an intracellular site, since they are too large to enter into a cell. There are many diseases for which there are unmet medical needs, which should be cured if gene expression could be controlled to the normal state. To achieve this goal, nucleic acid drugs are good candidates for reducing abnormal gene expression levels to the normal level, since they can interact specifically with mRNA in the cytosol or with DNA in the nucleus or mitochondria. However, nucleic acid drugs are usually negatively charged and have high molecular weight, which essentially prevents them from entering a cell, similar to an antibody. It is now evident that intelligent carrier systems are required for nucleic acids to reach the site of action and perform their action.

We have been developing a novel drug delivery system for use in controlling, not only the biodistribution but also the intracellular trafficking of a carrier system. We refer to this type of device as a multifunctional envelope-type nanodevice (abbreviated as MEND) (Nakamura, Akita, Yamada, Hatakeyama, & Harashima, 2012). Typically, our system has a core–shell structure where functional nucleic acids are condensed as the core which is covered with a lipid envelope, analogous to an envelope-type virus. To control pharmacokinetics as well as intracellular trafficking such as endosomal escape, nuclear or mitochondrial delivery, nanodevices can be introduced into the system to function based on the programmed delivery of nucleic acids to the site of action by overcoming barriers in our body (Akita, Hatakeyama, Khalil, Yamada, & Harashima, 2011). Once introduced into the blood circulation, nanocarriers are recognized by the immune system such as the complement system or antibodies. They need to escape cellular uptake in liver or spleen to reach the target tissue. Vascular endothelial cells are also a formidable barrier when we deliver nanocarriers to target tissues, except the liver, spleen and tumors which are sufficiently leaky to permit these nanocarriers to pass through the cell membrane. Once they reach the target cells by escaping endothelial barriers, the cellular membrane is the next barrier. In most cases, endocytosis is the major route for nanocarriers to enter into cells (Mudhakir & Harashima, 2009); however, it is essential

that they escape from endosomes to reach the site of action. Several classes of viruses have acquired intelligent molecular mechanisms that permit them to escape from the endosomal trap by responding the acidification in endosomes. The influenza virus can escape from endosomes via membrane fusion while adenovirus accomplishes this by membrane disruption. We are inspired by these fantastic mechanisms which viruses have acquired through evolution. Our systems have been improved as more information concerning the molecular mechanisms of viruses has appeared as well as by advances in molecular cell biology.

In this chapter, we summarize our studies on the development and improvement of various types of MENDs and discuss the possibilities and limitations of our system as they proceed to clinical use.

2. R8-MEND

2.1 Octaarginine (R8) Peptide

It is not surprising that the discovery of short peptides, which can translocate through cell membranes and transfer large biologically active molecules to the interior of cells, has attracted considerable attention in the field of drug and gene delivery (Derossi, Chassaing, & Prochiantz, 1998; Dietz & Bahr, 2004; Gupta, Levchenko, & Torchilin, 2005; Lindgren, Hallbrink, Prochiantz, & Langel, 2000; Schwarze, Ho, Vocero-Akbani, & Dowdy, 1999; Schwarze, Hruska, & Dowdy, 2000; Snyder & Dowdy, 2004; Trehin & Merkle, 2004). These peptides are generally known as cell-penetrating peptides (CPPs), membrane translocation sequences, "Trojan peptides" or protein transduction domains (PTDs), consist of 7–30 amino acids and have a net positive charge. The importance of these peptides in drug discovery lies mainly in their ability to carry massive cargos, several times larger than their own size, to the interior of a cell. Some of the commonly used CPPs are the transcriptional activator of transcription (TAT) of human immunodeficiency virus 1, penetratin which is the Antennapedia homeodomain 43–58 peptide and VP22 which is a transcription factor of Herpes simplex virus type I (Khalil, Kogure, Futaki, & Harashima, 2008). The CPPs share the presence of several arginine residues in their sequences and these arginine residues have been shown to be responsible for their activities (Futaki, Suzuki, et al., 2001; Vives, Brodin, & Lebleu, 1997; Wender et al., 2000). Peptides consisting of only arginine resides are highly active in terms of membrane translocation (Futaki, Suzuki, et al., 2001). The optimum number of arginine residues was shown to be 8, as evidenced by the activity

of octaarginine (R8) (Futaki, Suzuki, et al., 2001; Suzuki et al., 2002). The activities of longer or shorter oligoarginine are reduced. The R8 peptide was able to deliver exogenous protein into cells (Futaki et al., 2004). The delivery of an apoptotic-inducing peptide was accomplished by conjugation with R8 and the conjugate induced apoptosis in HeLa cells (Futaki et al., 2004). The R8 peptide was also used in the delivery of nucleic acids. The R8 peptide was able to transfect cells with a plasmid DNA as efficiently as polyarginine or polylysine (Futaki, Ohashi, et al., 2001). These results indicate that the R8 peptide is highly efficient in enhancing the cellular delivery of functional molecules.

The R8 peptide is able to control the intracellular trafficking of liposomes by modifying the liposomal surface with the R8 peptide. The *N*-terminal of R8 was modified by attaching a stearyl group to form stearylated R8 (STR-R8). STR-R8 is mixed with lipids when liposomes are formed. The hydrophobic stearyl moiety is expected to be anchored on the lipid bilayer of the liposome, resulting in presentation of the R8 peptide on the liposomal surface. The modification of liposomes with STR-R8 drastically enhanced the cellular uptake of a model drug encapsulated in the aqueous core of the liposome, compared to the use of the free drug or when the drug was encapsulated in liposomes without STR-R8 (Khalil, Kogure, Futaki, & Harashima, 2006; Khalil, et al., 2008). It is likely that cell surface proteoglycans such as heparan sulfate proteoglycans (HSPGs) are involved in the cellular binding and internalization of STR-R8-modified liposomes (R8-Lip) similar to other CPPs, because the presence of heparin almost completely inhibited the binding and uptake of R8-Lip (Khalil, Kogure, Futaki, et al., 2006). HSPGs probably function as nonspecific receptors for the binding of CPPs, which may explain why efficient binding occurs in most cell lines since HSPGs are present in nearly all cell lines (Belting, 2003; Yoneda & Couchman, 2003). The potential of the R8 peptide for efficient delivery has been an important stimulus in applications for gene delivery.

2.2 Construction of the R8-MEND

The R8-modified MEND (R8-MEND) is a first generation MEND. The strategy for producing the R8-MEND is based on forming a core–shell structure, which resembles an envelope-type virus (Kogure, Akita, & Harashima, 2007; Kogure et al., 2004). The R8-MEND consists of a condensed DNA core and a lipid membrane structure decorated with the R8 peptides (Figure 6.1(A)). DNA condensation allows the DNA to be protected from DNase, size control, and improved packaging efficiency. The method

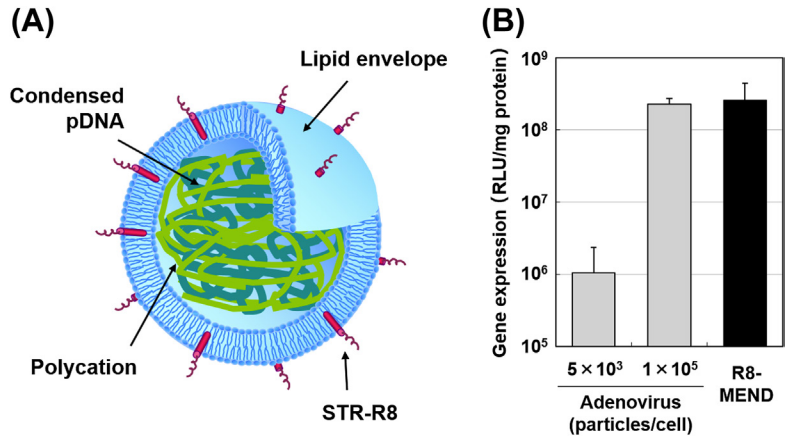

Figure 6.1 (A) Schematic representation of the R8-MEND. The R8-MEND consists of condensed pDNA and a lipid envelope containing STR-R8. (B) Gene expression in HeLa cells transfected with R8-MEND and adenovirus. *These figures are reproduced from Khalil et al. (2007).*

for constructing the R8-MEND is as follows. Plasmid DNA (pDNA) is first condensed electrostatically with a polycation such as poly-L-lysine (PLL), protamine, or STR-R8 under vortexing (Kogure et al., 2004; Moriguchi et al., 2005). The size and charge of the condensed DNA is controlled by the ratio of pDNA and polycation. Furthermore, the ratio influences the size, charge, and encapsulation efficiency. The lipid film containing a negatively charged lipid such as cholesteryl hemisuccinate (CHEMS), phosphatidic acid (PA) or cardiolipin (CL) is hydrated with the pDNA/polycation particles (Akita, Kogure, et al., 2010; Akita et al., 2009; Kogure, et al., 2004; Moriguchi, et al., 2005). The packaging of pDNA/polycation particles with the lipid bilayer is performed by sonication. The negatively charged MEND is then formed. Finally, STR-R8 solution is added to the negatively charged MEND. The diameter of the resulting R8-MEND is 100–300 nm and is highly positively charged.

2.3 R8-MEND for Gene Delivery

The potential of the R8-MEND as a gene delivery system is introduced in this section. Positively charged pDNA/PLL particles showed a higher reporter gene expression compared to naked pDNA in NIH3T3 cells (Kogure, et al., 2004). A MEND formulation of pDNA/PLL particles showed transfection activity that was 10-fold higher than that for pDNA/PLL particles. Moreover, the R8-MEND drastically enhanced gene expression level by

1000-fold over that of pDNA/PLL particles. The transfection activity of the R8-MEND was compared with that of an adenovirus, one of the strongest viral vectors, in a human cervical cancer cell line HeLa and in a human lung epithelial carcinoma cell line A549 (Khalil et al., 2007). The transfection activities of the R8-MEND were comparable to those of adenovirus using 1×10^5 particles/cell (Figure 6.1(B)). Moreover, no significant toxicity was observed in the case of the R8-MEND treatment, although adenovirus induced a significant cytotoxicity.

The R8 density on the surface of the MEND has a great influence on transfection activity. A MEND modified with a high density of R8 (R8-MEND-HD) showed a sevenfold higher cellular internalization than that of the low density of R8-modified MEND (R8-MEND-LD) (Khalil, Kogure, Futaki, et al., 2006). On the other hand, the transfection activity of R8-MEND-HD was 1000-fold higher than that of the R8-MEND-LD. The superiority of the R8-MEND-HD regarding gene expression cannot be explained by differences in the amount of pDNA taken up into the cells, indicating that intracellular events are responsible for this difference. Confocal laser scanning microscopy (CLSM) observations of the R8-MEND-HD and the R8-MEND-LD clearly showed a significant difference in intracellular fate (Khalil, Kogure, Futaki, et al., 2006). The R8-MEND-LD is substantially colocalized with lysosomes, suggesting that the R8-MEND-LD is degraded in lysosomes. On the other hand, only a partial colocalization of the R8-MEND-HD in lysosomes was found, indicating that the R8-MEND-HD efficiently avoided lysosomal degradation. The difference in intracellular trafficking between the R8-MEND-HD and the R8-MEND-LD can be explained by the difference in the internalization mechanism (Khalil, Kogure, Futaki, et al., 2006). The cellular uptake of the R8-MEND-LD was strongly inhibited by a hypertonic medium, whereas these conditions inhibited the cellular uptake of R8-MEND-HD by only about 35%. This indicates that clathrin-mediated endocytosis is the major uptake pathway for the R8-MEND-LD. On the other hand, the cellular internalization of R8-MEND-HD was strongly inhibited by approximately 80% in the presence of a macropinocytosis inhibitor amiloride, although the inhibition of the cellular uptake of the R8-MEND-LD was around 40%. This suggests that the R8-MEND-HD is mainly taken up by cells via macropinocytosis. The intracellular fate of the R8-MEND internalized via clathrin-mediated endocytosis or macropinocytosis is an important issue. Particles internalized via clathrin-mediated endocytosis eventually undergo degradation

in the lysosomes, while macropinosomes do not extensively fuse with the lysosomes in most cell types (Khalil, Kogure, Akita, & Harashima, 2006). Thus, macropinocytosis is the preferred uptake pathway for gene delivery. Actually, the blocking of macropinocytosis inhibited gene expression mediated by R8-MEND-HD by around 95% (Khalil, Kogure, Futaki, et al., 2006). Collectively, the density of R8 on the surface of the MEND determines the intracellular fate of R8-MEND and the transfection activity.

Modification of the surface of the MEND with R8 enhances endosomal escape, in addition to stimulating macropinocytosis. The process of endosomal escape is crucial for achieving efficient gene delivery. The role of surface modification with R8 on escape from endocytic vesicles was investigated by using octalysine (K8) as a control cationic peptide (El-Sayed, Khalil, Kogure, Futaki, & Harashima, 2008). The gene expression of R8-MEND was 17-fold higher than that of the K8-modified MEND (K8-MEND). However, the physicochemical characteristics of the R8-MEND and K8-MEND were comparable, which could not explain the difference in gene expression level. Furthermore, the extent and rate of pDNA delivered by the R8-MEND was similar to that for the K8-MEND. The uptake pathway (macropinocytosis) for both MENDs was also the same. Thus, the difference in gene expression between the R8-MEND and the K8-MEND cannot be attributed to differences in the cellular uptake of the MEND. On the other hand, CLSM observations clearly showed that R8-MEND escaped more efficiently from endosomal vehicles compared to the K8-MEND. A quantitative analysis of CLSM images revealed that the extent of R8-MEND escape from endosomes was also higher than that of the K8-MEND, 62% compared with only 26% for the K8-MEND. Interestingly, the endosomal escape of the K8-MEND was enhanced at a less acidic endosomal pH mediated by treatment with bafilomycin A1, a selective vacuolar proton pump inhibitor, whereas that of the R8-MEND was inhibited. The destabilization assay of artificial membrane showed that R8-MEND fused efficiently at both acid and neutral pH, although the K8-MEND fused only at a neutral pH. Collectively, the R8 peptide promotes endosomal escape via a fusion mechanism that is functional at both neutral and acidic pH.

In the above section, we concluded that the gene expression of the R8-MEND was comparable to that of adenovirus. Viruses, such as adenovirus, have developed sophisticated machinery for efficient infection, namely the delivery of their own gene to the nucleus (Mudhakir & Harashima, 2009).

A comparison of cytoplasmic transport between the R8-MEND and adenovirus was performed (Akita, Enoto, et al., 2010; Nakamura et al., 2012). The transfection activity of the R8-MEND was drastically decreased in the presence of nocodazole, a microtubule disrupting reagent. The tracking of the R8-MEND and adenovirus by multicolor real-time imaging demonstrated that the R8-MEND as well as adenovirus was subject to directional transport along with the microtubules. Adenovirus becomes directly associated with motor proteins. However, interestingly, a portion of the R8-MEND was transported together with endosomes, while the extent of colocalization of adenovirus with endosomes was negligible. That is, the vesicular trafficking is involved in the transport of R8-MEND. Collectively, these data clearly indicate that the cytosolic transport of the R8-MEND is completely different from that of adenovirus.

Gene delivery to hair follicles is an attractive approach for the treatment of skin and hair disorders (Domashenko, Gupta, & Cotsarelis, 2000; Li & Hoffman, 1995; Saito et al., 2002). The potential of the R8-MEND for in vivo gene therapy for hair and skin disorders was recently tested (Khalil et al., 2007). R8-MEND containing pDNA coding for lacZ was applied to the dorsal skin of mice and lacZ activity was measured in the tissue sections by a colorimetric assay for β-galactosidase. β-Galactosidase activity was observed in hair follicles of mice treated with R8-MEND (Figure 6.2(A)). The transfection efficiency of the R8-MEND was approximately 24%. In contrast, when the same experiment was performed with Lipofectamine, the transfection efficiency was only 0.2% (Figure 6.2(A)). These data suggest that the R8-MEND may be useful for delivering therapeutic drugs to hair follicles. Thus, the BMP receptor type 1A (BMPR1A) gene, an essential gene in regulating hair cycling (Kobielak, Pasolli, Alonso, Polak, & Fuchs, 2003; Yuhki et al., 2004), corded pDNA was incorporated into an R8-MEND and the effect of transfection mediated by R8-MEND was investigated in the hair cycle (Khalil et al., 2007). The hair follicles were deeper in the subcutis space in mice that had been treated with the R8-MEND than in control mice. A fraction of hair follicles in the mice treated with R8-MEND was in the late anagen or early catagen phase, although hair follicles in control mice were in the late catagen phase or telogen phase. Moreover, cell proliferation was detected 2 weeks after treatment in hair follicles exposed to the R8-MEND, but not in the control hair follicles. These facts clearly indicate that the R8-MEND is a potent carrier for delivering genetic material to hair follicles.

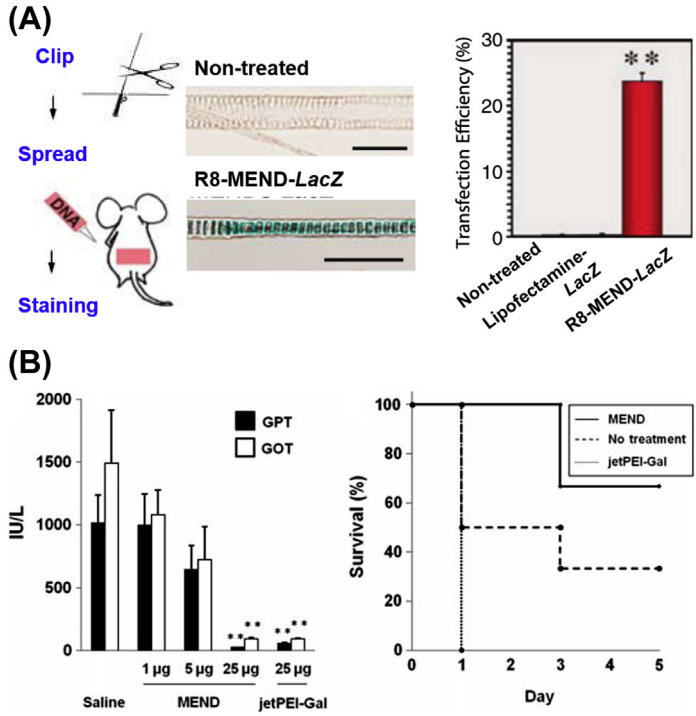

Figure 6.2 (A) Hair form the dorsal skin of mice was clipped and skin was treated with an R8-MEND containing LacZ-expression pDNA. The X-gal staining of hairs from controls or R8-MEND treated skins is shown. Transfection efficiency of R8-MEND versus Lipofectamine. $^{**}P < 0.001$; control versus R8-MEND, Lipofectamine versus R8-MEND. *(These figures are reproduced from a literature reference Khalil et al. (2007).)* (B) GPT and GOT values in serum from mice treated with R8/GALA-MEND containing HGF pDNA and in vivo jetPEI-Gal. $^{**}P < 0.01$ versus mice group treated with saline. *(These figures are reproduced from Hayashi, et al. (2012).)*

Intravenous administration is a typical method for gene delivery to a variety of tissues. R8-modified liposomes largely accumulate in the liver after intravenous administration and this is dependent on the density of the R8 peptide on liposomal surface (Mudhakir, Akita, Khalil, Futaki, & Harashima, 2005). The use of the R8-MEND in gene delivery to the liver by intravenous administration was tested (Hayashi, Mizuno, Ikramy, Akita, & Harashima, 2012; Khalil, Hayashi, Mizuno, & Harashima, 2011). Modification of the R8-MEND with the GALA peptide and the use of negative pDNA core are required for gene expression in the liver (Khalil et al., 2011). GALA peptide was originally developed as an endosomal destabilizer, inspired by the mechanism of HA2 in the influenza virus (Li,

Nicol, & Szoka, 2004). The GALA peptide adopts an α-helical conformation in the acidic pH in endosomes. Conjugation of cholesterol to the GALA peptide allows anchoring to the lipid bilayer so that the GALA peptide becomes spontaneously oriented outward from the surface of the envelope of the MEND (Kakudo et al., 2004). Gene expression in the liver after the intravenous administration of the R8-MEND was very low and the difference between the R8-MEND with a positive pDNA core and a negative pDNA core was negligible in the absence of the GALA peptide. On the other hand, a GALA modified R8-MEND (R8/GALA-MEND) showed a drastic enhancement in liver gene expression by 1650-fold in the case of the R8/GALA-MEND with a negative pDNA core, while only a threefold improvement was observed in the case of the R8/GALA-MEND with a positive pDNA core. Contrary to our expectations, quantification of the number of gene copies delivered to liver cells and nuclei by the MEND revealed that the amount of pDNA was significantly higher in the case of R8-MEND with a positive pDNA core, regardless of the absence or presence of the GALA peptide. This finding suggests that the substantial improvement in gene expression in the liver by the R8/GALA-MEND with a negative pDNA core can be attributed to an improved gene expression efficiency per pDNA in the presence of the GALA peptide. Furthermore, we examined the issue of whether the R8/GALA-MEND exerted potent hepatoprotective effects against a lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced acute liver injury (Hayashi, et al., 2012). Treatment with LPS and D-GalN induces the over production of tumor necrosis factor α from activated macrophages and blocking of the nuclear factor κB induced the survival of gene expression, resulting in acute liver damage and an increase in glutamate pyruvate transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) levels in serum. An R8/GALA-MEND loading hepatocyte growth factor (HGF) expressing pDNA was prepared and the positive protective effect of the R8/GALA-MEND was compared with that of in vivo transfection reagent (jetPEI^{TM}-Gal) which is a well-known commercially available reagent for delivering genetic material to the liver. The treatment with the R8/GALA-MEND loading HGF pDNA significantly decreased the serum levels of GOT and GPT, while the effect was comparable to that of in vivo jetPEI-Gal (Figure 6.2(B)). On the other hand, R8/GALA-MEND treated mice group showed a significantly higher survival rate than the in vivo jetPEI-Gal treated mice group (Figure 6.2(B)). Two mechanisms are considered for improving acute liver injury by the

administration of R8/GALA-MEND loading HGF pDNA. The first is that HGF pDNA delivered to the liver induces an antiapoptotic effect through the dual induction of Bcl-xL and COX2, which suppresses caspase-3 activity (Nomi, Shiota, Isono, Sato, & Kawasaki, 2000). The second is that the expressed HGF exerts a strong mitogenic action for hepatocytes, which promotes liver regeneration (Fujiwara et al., 1993; Ishiki, Ohnishi, Muto, Matsumoto, & Nakamura, 1992; Roos, Terrell, Godowski, Chamow, & Schwall, 1992) and stimulates the synthesis of hepatic protein responsible for liver-specific functions (Matsuda et al., 1997; Takehara, Matsumoto, & Nakamura, 1992). Collectively, R8/GALA-MEND represents a promising nonviral vector for liver gene delivery and for preventive action regarding hepatic failure.

2.4 Extended Applications of the R8-MEND

The R8-MEND is able to apply to deliver functional molecules other than pDNA. We introduce the extended application of R8-MEND such as the delivery of small interfering RNA (siRNA), protein/lipid antigens, and adjuvants.

siRNA can silence specific genes by RNA interference (RNAi), which is considered to be a powerful tool for research and medical care. The delivery of siRNA to the cytosol of cells is required to induce the efficient production of RNAi, because RNAi is induced by loading the RNA-induced silencing complex (RISC) with siRNA in the cytosol (Wilson & Doudna, 2013). The reader is directed to the relevant chapters (Pieter R. Cullis et al.) of this book. Efficient cytosolic delivery of siRNA was expected by incorporating siRNA into R8-MEND. The R8-MEND loaded with siRNA showed higher gene silencing than TransIT-TKO, a commercially available delivery reagent, in HeLa cells (Nakamura, Kogure, Futaki, & Harashima, 2007). CLSM observations indicted that the R8-MEND efficiently delivered siRNA to the cytosol. The R8-MEND was found to have a strong gene knockdown effect (>75%) against HeLa-GL3 cells at a concentration of approximately 120 nM (Akita, Kogure, et al., 2010). However, when the dose of siRNA was decreased, gene knockdown effect gradually decreased, with an IC^{50} of approximately 75 nM, and eventually decreased to >10% at 1/10 of the original dose. A quantitative imaging analysis of the intracellular trafficking of siRNA revealed that the dissociation process, as well as the rate of endosomal escape limits the RNAi efficiency of the R8-MEND. Thus, we upgraded the R8-MEND in a stepwise manner based on the analysis of intracellular

trafficking (Akita, Kogure, et al., 2010). Successful endosomal escape was achieved by modification with the GALA peptide that was optimized for endosomal fusion. Furthermore, a modified protocol for the preparation of the R8-MEND, mixing siRNA core and small unilamellar vesicles (SUV), resulted in a more homogenous, smaller diameter particle, and resulted in a more efficient intracellular dissociation. This optimized R8-MEND (R8/GALA-MEND$_{SUV}$) resulted in a drastic increase in the gene knockdown effect to more than 70%, even at a dose of 12 nM (1/10 dose). Moreover, the R8/GALA-MEND$_{SUV}$ was applied to dendritic cells (DC) therapy (Akita, Kogure, et al., 2010). The suppressor of cytokine signaling 1 (SOCS1) is a negative-feedback regulator of immune cell responses to cytokines (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). The expression of SOCS1 is induced by cytokine stimulation, and is responsible for the negative-feedback of immune responses by blocking JAK-STAT signaling. The knockdown of the SOCS1 gene in DCs using a lentiviral vector has been shown to enhance their immunostimulatory capacities and to result in enhanced antiviral and antitumor responses (Shen, Evel-Kabler, Strube, & Chen, 2004; Song et al., 2006). However, the use of a viral vectors may have unexpected and serious side effects such as excessive inflammation, and oncogenicity due to the random insertion of viral DNA into chromosomal DNA (Hacein-Bey-Abina, von Kalle, Schmidt, Le Deist, et al., 2003; Raper et al., 2003). Hence, nonviral delivery systems, which satisfy the requirements for both high efficiency and safety, are essential for realizing the clinical applications of siRNA (Zhang, Zhao, Jiang, Wang, & Ma, 2007). R8/GALA-MEND$_{SUV}$ loaded with anti-SOCS1 siRNA was prepared and the knockdown effect against SOCS1 gene was investigated in mouse bone-marrow-derived DCs (BMDCs) (Akita, Kogure, et al., 2010). Treatment of R8/GALA-MEND$_{SUV}$ loaded with anti-SOCS1 siRNA reduced SOCS1 mRNA levels to 21.5% of the levels of nontreated BMDCs (Figure 6.3(A)). The SOCS1-silenced BMDCs were more responsive to IFN-γ than control BMDCs, as evidenced by the enhanced and prolonged phosphorylation of STAT1 (Figure 6.3(B)). The knockdown of the SOCS1 gene by R8/GALA-MEND$_{SUV}$ significantly promoted the production of TNF-α (Figure 6.3(C)) and IL-6. Furthermore, the administration of BMDCs transfected with anti-SOCS1 siRNA-induced strong antitumor effects against lymphomas compared to that of control siRNA (Figure 6.3(D)). These results clearly indicate that the R8/GALA-MEND$_{SUV}$ is a powerful tool for delivery of siRNA to DCs.

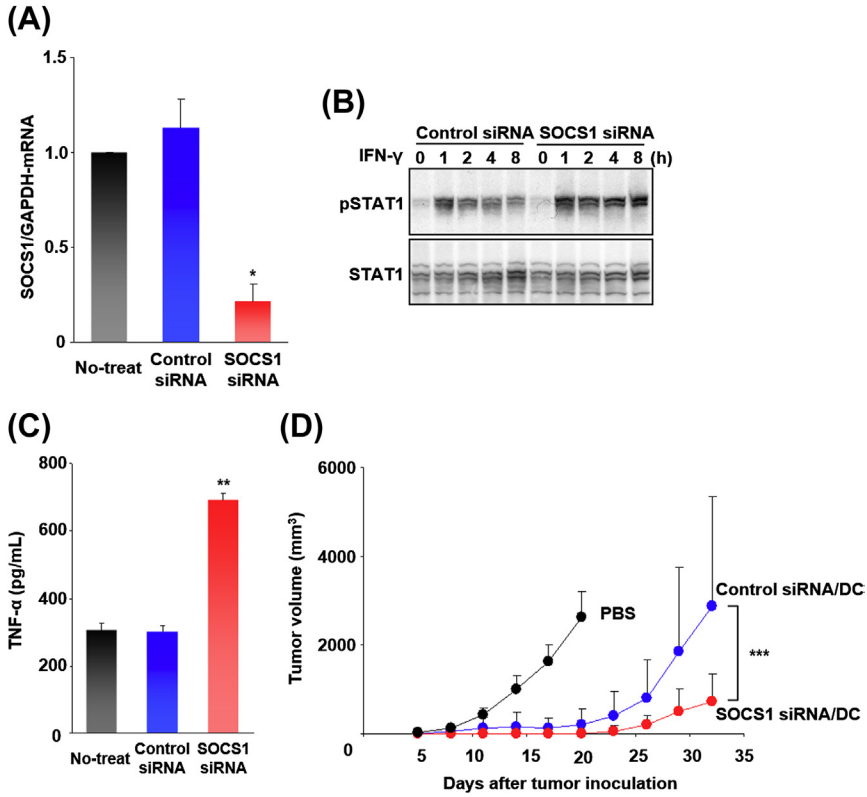

Figure 6.3 (A) SOCS1 mRNA levels in BMDCs transfected with R8/GALA-MEND$_{SUV}$ loaded with anti-SOCS1 siRNA or control siRNA *$P < 0.05$ versus control siRNA. (B) STAT1 tyrosine phosphorylation in anti-SOCS1 siRNA or control siRNA transfected BMDCs. (C) TNF-α concentration in culture supernatants of BMDCs transfected with R8/GALA-MEND$_{SUV}$ loaded with anti-SOCS1 siRNA or control siRNA at 24 h after stimulation with IFN-γ. **$P < 0.01$ versus nontreated BMDCs. (D) Inhibition of E.G7-OVA tumor growth by SOCS1-silenced BMDCs immunization. ***$P < 0.005$ versus control siRNA/DC. *These figures are reproduced from Akita, Kogure, et al. (2010).*

A cancer immune therapy is expected as the fourth generation of cancer therapy after surgery, chemotherapy, and radiotherapy. Cellular immunity is indispensable for the efficient elimination of tumors. In antitumor immunity, cytotoxic T lymphocytes (CTL) mainly function as terminators against tumor cells. The efficient activation of CTL is required for cytokines and antigen presentation via the major histocompatibility complex I (MHC-I) on antigen presenting cells (APCs). MHC-I antigen presentation is induced by the degradation of cytosolic antigens via proteasomes. It is necessary to deliver tumor associated antigens to the cytosol of APCs, because the

tumor associated antigens used in cancer immunotherapy are basically exogenous. The characteristics of the R8-MEND, high cellular delivery and endosomal escape, make them suitable for antigen delivery. R8-MEND encapsulating ovalbumin (R8-MEND/OVA) showed a highly specific MHC-I antigen presentation in BMDCs, which could be due to the efficient cytosolic delivery of encapsulated substances (Nakamura, Moriguchi, Kogure, Shastri, & Harashima, 2008). Mice immunized with R8-MEND/OVA showed preventive antitumor effects against E.G7-OVA tumors. Furthermore, the coencapsulation of polyinosine-polycytidylic acid (poly I:C) into R8-MEND/OVA drastically facilitated antitumor immunity such as CTL activity, preventive and therapeutic antitumor effects (Nakamura, Moriguchi, Kogure, & Harashima, 2013). Poly I:C is an adjuvant and activates APCs via the Toll-like receptor 3 (TLR3) and the melanoma differentiation-associated gene 5 (Akira, Uematsu, & Takeuchi, 2006). The enhancement of antitumor immune responses failed when a complex of R8, OVA, and poly I:C was used. This fact indicates the importance of topological control based on programmed packaging in antigen/adjuvant delivery.

The development of an R8-MEND based tuberculosis vaccine and a bladder cancer vaccine are ongoing. The prominent cellular delivery by R8-MEND is a key technology for these applications.

We now know that a new paradigm exists for antigen presentation, which involves the recognition of nonpeptide antigens present on CD1 molecules by T cells (Sugita, Cernadas, & Brenner, 2004). CD1 molecules bind lipids such as foreign fatty acids, glycolipids, and lipopeptide antigens different from self-origin (Barral & Brenner, 2007). These lipid antigens are mainly found in bacteria, particularly *mycobacterium*. We found that glucose monomycolate (GMM), derived from mycobacteria, functioned as an antigen and was able to elicit a delayed-type hypersensitivity against *mycobacterium* in guinea pigs, which was achieved using an R8-MEND incorporating GMM (Komori et al., 2011). The R8-MEND incorporating GMM also induced GMM-specific T-cell responses and Th1-skewed tissue responses in the mycobacteria-infected rhesus macaques (Morita, Hattori, et al., 2013; Morita, Miyamoto, et al., 2013). These results indicates that an R8-MEND incorporating GMM can function as a potent vaccine against tuberculosis.

The intravesical instillation of live *Bacillus* Calmette-Guerin (BCG) is used in the treatment of superficial bladder cancer and carcinoma in situ, in addition to a vaccine against tuberculosis (Redelman-Sidi, Glickman, & Bochner, 2014). Although BCG therapy is a very effective therapy, serious side effects associated with the use of live mycobacteria pose a significant concern (Lamm et al., 1992).

It is essential to develop a noninfectious and less toxic immunotherapeutic drug. The BCG cell wall skeleton (BCG-CWS) would be a potent substitute for live BCG. However, because BCG-CWS has unfavorable physical characteristics, such as poor solubility and a very high molecular weight, it cannot be used in a drug formulation. BCG-CWS in hydrophilic solution shows heterogeneity in the size of tens of micrometers (Uenishi, Kawabe, Nomura, Nakai, & Sunagawa, 2009). These unfavorable characters hamper the cellular delivery of BCG-CWS to target cells such as bladder cancer cells and APCs. However, we succeeded in developing a novel packaging method, the liposome evaporated via emulsified lipid method, that permit BCG-CWS to be encapsulated into a sub-200-nm-sized R8-MEND (Nakamura et al., 2014). The R8-MEND encapsulating BCG-CWS (R8-MEND/CWS) showed a high uniformity and can be easily dispersed in water. In functional evaluations, R8-MEND/CWS efficiently delivered BCG-CWS to mouse bladder cancer cells and inhibited tumor growth in mice bearing mouse bladder tumors (Nakamura et al., 2014). Moreover, the intravesical administration of R8-MEND/CWS resulted in the shrinking of bladder tumors in a carcinogen-induced urinary bladder cancer rat model, indicating that R8-MEND/CWS is fully capable of delivering BCG-CWS to bladder cancer cells and APCs in the intravesical environment. Since the rat model is similar to the actual clinical condition, R8-MEND/CWS is expected to be a valid candidate for a clinical immunotherapeutic drug against bladder cancer.

2.5 Perspectives of the R8-MEND

The R8-MEND, a first generation of MEND, represented a potential strategy for the delivery of functional molecules such as pDNA, siRNA, antigens, and adjuvants to the cells. The next generation of MEND for pDNA and siRNA delivery is being developed based on the insights obtained by using of R8-MEND. On the other hand, R8-MEND based technologies such as R8-MEND/GMM and R8-MEND/CWS appear to be viable clinical drug candidates.

3. KALA-MEND

3.1 A Multicoated MEND as a Strategy for Intracellular Trafficking via Stepwise Membrane Fusion

The treatment or prevention of disease by gene therapy represents a potential revolution in disease therapy. The development of a DNA vaccine is one of the challenges for the cure of cancer and a variety of infectious

diseases. To successfully develop a DNA vaccine, innovative nanotechnology designed to confer the effective expression of the antigen-encoding genes in DCs is prerequisite, since these cells play crucial roles in the initiation and regulation of innate and adopted immune responses (Joffre, Segura, Savina, & Amigorena, 2012). However, gene delivery targeting DCs is generally hampered by cellular membrane barriers. The nuclear membrane is the ultimate barrier to overcome in nondividing DCs, since the nuclear region is separated by a double lipid bilayer structure. Moreover, the nuclear pore complex (NPC) exclusively controls the mutual transport of large sized (>39 nm) (Pante & Kann, 2002) and/or hydrophilic macromolecules (Kumeta, Yamaguchi, Yoshimura, & Takeyasu, 2012) between the cytoplasm and the intranuclear compartment.

Various types of viral vectors, such as the adenovirus (Dietz & Vuk-Pavlovic, 1998; Ribas, 2005), have been used for the foreign transgene expression in DCs, since viral vectors are typically more effective than nonviral ones. However, the use of viral vectors is accompanied by some risks associated with the immunogenic (Marshall, 1999) and/or carcinogenic characteristics of such systems (Hacein-Bey-Abina, Von Kalle, Schmidt, McCormack, et al., 2003; McCormack & Rabbitts, 2004; Thomas, Ehrhardt, & Kay, 2003). Based on these drawbacks, the development of nonviral vectors would be highly desirable. Concerning an artificial approach, certain types of physical methods including electroporation have been reported for gene delivery targeting DCs (Davtyan et al., 2014). However, the necessity of using a physical device may pose a barrier to medical institutions while a DNA vaccine approach may not. Thus, an innovative nanotechnology capable of introducing exogenous genes to DCs without using a viral carrier or a physical device would be desirable.

Our laboratory previously attempted to apply the R8-MEND as a gene delivery system targeting DCs. While it exhibited high gene expression in dividing cells (Khalil et al., 2007; Kogure et al., 2007), it failed to achieve gene expression against BMDCs (Nakamura et al., 2006). Since DCs are not dividing to any extent, being much slower than culture cells (i.e., HeLa cells), we assumed that the nuclear membrane was the major factor in limiting the nuclear entry of pDNA. To overcome the endosome and nuclear membranes in a stepwise manner, we developed a tetralamellar multifunctional envelope-type nanodevice (T-MEND), in that the key structural elements of which are a DNA/polycation condensed core that is encapsulated by two types of lipid envelopes; two nuclear membrane-fusogenic inner envelopes, and endosome-fusogenic outer envelopes (Akita et al., 2009).

The membrane fusion activity of liposomes to the nuclear membrane was assessed by fluorescence resonance energy transfer (FRET). Various compositions of liposomes were labeled with both 1 mol% 7-nitrobenz-2-oxa-1,3-diazole (NBD)-DOPE (1, 2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine) and 0.5 mol% rhodamine-DOPE and were incubated with isolated nuclei. Membrane fusion between the labeled liposomes and isolated nucleus would lead to the diffusion of NBD and rhodamine into the lipid membranes, which causes the release of FRET. Screening the liposomes by FRET analysis revealed that a combination of DOPE and CL have a highly potent in-fusion activity with reference to the nuclear membrane. When isolated nuclei were incubated with green fluorescence protein (GFP)-encapsulated and rhodamine-labeled multilamellar vesicle liposomes prepared with nucleus-fusogenic lipids, GFP was detected inside the nucleus whereas labeled lipids were distributed along the nuclear membrane. Alternatively, GFP and lipid labeling was colocalized along the nuclear membrane when the liposomal composition was altered to that of the nonfusogenic one (EPC/Chol). These data indicated that nuclear transport of macromolecules can be achieved only when it is encapsulated into the multilayered liposomes composed of nucleus-fusogenic lipids. In addition, gene transfection efficiency with a multilamellar MEND, composed of a nucleus-fusogenic inner envelope, and an endosome-fusogenic outer envelope represented a significantly higher transfection activity in mouse dendrite-derived cell line (JAWS II) in comparison with a conventional R8-MEND, while the level of gene expression was not sufficient to reach a level sufficient to achieve antigen presentation.

3.2 Induction of Transfection Activity by Modification with KALA

For upgrading the T-MEND, we attempted to improve the fusogenic activities of the endosome-fusogenic lipid and nuclear membrane-fusogenic lipids. In this case, a peptide that possessed fusogenic activity in a physiological environment would be desired for inducing fusion to the nuclear membrane since the environment in the cytoplasm is neutral. Thus, we used KALA, an α-helical peptide that induces membrane fusion at a neutral pH (Wyman et al., 1997). For the spontaneous orientation of the KALA peptide outward from the lipid envelope, we synthesized a lipid derivative of KALA. Cholesterol was used for anchoring of GALA. However, it is also known that cholesterol functions as a lipid membrane stabilizer, and thereby could potentially inhibit the membrane fusion process. Thus, we alternatively

employed a stearyl moiety for this anchoring. The original KALA also contains a cysteine residue at its C-terminus (Wyman et al., 1997). Therefore, KALA may form a disulfide-bonded dimer, and then could hamper its fusion activity. We thus employed a truncated version of KALA that lacks the three C-terminal residues (STR-KALA: stearyl-WEAKLAKALAKAL-AKHLAKALAKALKA-amide). By systematic optimization, we found that modification of STR-KALA with 5% in the inner envelope and 1.5% in the outer one are the optimum combination for achieving efficient gene expression. Furthermore, reducing the charge (+/−) ratio of the pDNA/polycation core particle from 5 to 0.5 also improved transfection activity. The combination of these optimized modification of STR-KALA (1.5% and 5% of STR-KALA in outer or inner envelope, and a negatively charged core particle) and a reduction in the amount of polycation in the T-MEND design resulted in a prominent increase in transfection efficiency by 30-fold compared with that for the conventional T-MEND. The level of gene transfection reached a sufficient level to produce significant antigen presentation (Shaheen, Akita, Nakamura, et al., 2011).

3.3 Immune-Stimulative Activity of KALA-Modified MEND

While the STR-KALA was originally designed in an attempt to improve the fusogenic activity of the lipid envelope of the T-MEND, we unexpectedly found that the transfection activity was drastically improved, even when STR-KALA was incorporated onto the simple architecture of the R8-MEND (R8/KALA-MEND); modification with STR-KALA drastically improved the transfection activity, depending on its density (at most > 2 orders of magnitude in 5% modification), while modification of the Chol-GALA resulted in only a marginal increase in gene expression (at most approximately fivefold at a 2% level of modification). To address the mechanism responsible for the drastic improvement in gene expression in the R8/KALA-MEND, the amount of pDNA taken up by cells, or delivered to the nucleus were quantitatively compared between the R8-MEND and the R8/KALA-MEND by quantitative PCR at 24 h after the transfection. However, the 2 orders of magnitude higher gene transfection activity for the R8/KALA-MEND cannot be explained by the intracellular trafficking processes. Therefore, it was strongly indicated that the high gene expression in R8/KALA-MEND can be attributed to the postnuclear delivery process (i.e., transcription), rather than intracellular trafficking processes (Akita, Ishii, et al., 2013). To gain further insights into the mechanism responsible for the stimulative transcription, the variation in the gene expression profile

in host cells (JAWS II cells) in response to the transfection of R8/KALA-MEND was assessed using a whole-genome oligonucleotide microarray. In this study, the JAWS II cells were transfected with the R8-MEND or the R8/KALA-MEND, and the mRNA was isolated at 6 h after transfection. The correlation of the expression levels of individual mRNA is shown in the form of a scattered plot, in which the gene expressions in the cells transfected with R8-MEND (A) or R8/KALA-MEND (B) (*Y*-axis) were plotted against those of nontreated cells (*X*-axis) (Figure 6.4). In the case

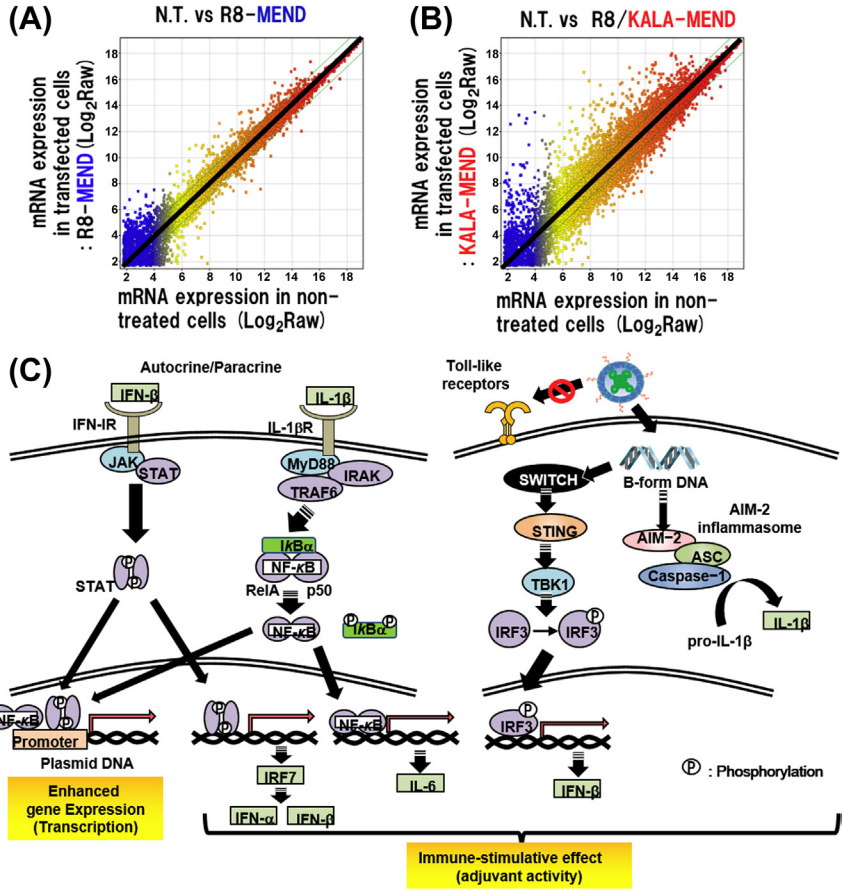

Figure 6.4 ***Activation of the dendritic cells by KALA-modified MEND (KALA-MEND).*** Scatter plot for the correlation of individual gene expression in JAWS II between nontreated cells and ones transfected with R8-MEND (A) or R8/KALA-MEND (B). *X*-axis and *Y*-axis represent the gene expression of individual genes in nontreated cells and MEND-transfected cells, respectively. (C) Schematic diagram illustrating a possible mechanism for stimulation of immune-response and enhanced transgene expression by means of KALA-MEND.

of the R8-MEND, major part of dots were located close to the 1-to-1 correlation line. Thus, mRNA expression was not perturbed even after the transfection with R8-MEND. In contrast, after transfection with the R8/KALA-MEND, a large amount of dots were located far from the 1-to-1 correlation line. Thus, the KALA-MEND might activate a certain kind of switch that triggers the signaling in DCs. In fact, when we counted the number of mRNA molecules whose expression was varied by more than three folds, it reached above >1100 out of 21,955 genes analyzed. To identify the transcription-stimulating pathway or factors that are responsible for the high gene expression in the R8/KALA-MEND, the extracted genes that were, in turn, categorized as transcription-related genes that were subjected to a pathway analysis. As a result, we unexpectedly found that the cascade ultimately falls into several transcription factors: STATs and NF-κB, which are well known to be activated when the immune-responsive cells were stimulated. In fact, a mode detail analysis of the microarray data indicated that the gene expression of cytokines or chemokines were also induced after transfection with the R8/KALA-MEND. Interestingly, the transfection activity (Akita, Ishii, et al., 2013) or cytokine production (unpublished observation) in response to the KALA-modified MEND in BMDCs was not abolished even when any of the TLRs were knocked-out. It is quite plausible that certain kinds of cytoplasmic DNA sensors that are related to the STING-TBK1 pathways, or inflammasome (Barber, 2011; Desmet & Ishii, 2012; Ishii et al., 2008; Ishikawa & Barber, 2008; Ishikawa, Ma, & Barber, 2009) are involved in the activation of the immune responses (unpublished observation).

4. MITO-PORTER

Mitochondria possess various essential organelle functions including ATP production and apoptosis regulation, and their own genome, mitochondrial DNA (mtDNA). It has recently become evident that a variety of human diseases are associated with mitochondrial dysfunction (Schapira, 2006; Tuppen, Blakely, Turnbull, & Taylor, 2010). It is now well accepted that mutations and defects in mtDNA form the basis of these diseases (Goto, Nonaka, & Horai, 1990; Shanske et al., 1990; Shoffner et al., 1990; Wallace, 1999). Therefore, mitochondrial gene therapy and diagnosis would be expected to have substantial medical benefits. The mitochondrion possesses a two membrane structure consisting of an outer membrane and an inner membrane, with an intermembrane space, where key components

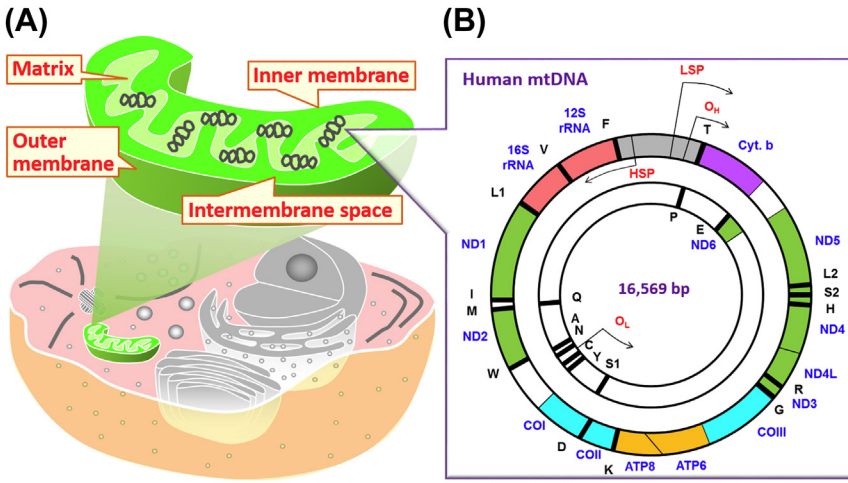

Figure 6.5 ***Mitochondrion and the mitochondrial genome (mtDNA).*** The mitochondrion possesses two membrane structures consisting of an outer membrane and an inner membrane, and the inner most is the matrix, which pools mitochondrial genome (mtDNA), while the space between the membranes is called intermembrane space (A). Map of human mtDNA (B). It contains 13 polypeptide-encoding genes, two ribosomal RNA, and 22 transfer RNAs (tRNAs). Single letter code is given for each tRNA-encoding gene. ND, NADH dehydrogenase coding subunits; CO, cytochrome oxidase coding subunits; ATP, F_1F_0-ATP synthase coding subunits.

of the electron transfer system are found, and a matrix, where mtDNA is pooled (Figure 6.5). Drugs can pass through the outer membrane if their molecular weight is less than 5 kDa, however, they cannot penetrate through the inner membrane which has tight membrane structure. To achieve mitochondrial gene therapy, it will be necessary to deliver functional agents into the mitochondrial matrix, over the barrier of both the cellular and mitochondrial double membranes. In this section, mitochondrial gene therapeutic strategies and the various strategies for delivering nucleic acids to mitochondria are reviewed. In particular, we discuss nucleic acid vectors for mitochondrial delivery. Our current efforts regarding the Dual Function (DF)-MITO-Porter, a liposome-based nanocarrier for mitochondrial delivery via a stepwise process, are also summarized.

4.1 Mitochondrial DNA and Mitochondrial Diseases

The human mitochondrion possesses its own genome (mtDNA), which is different from the nuclear genome, in the innermost mitochondrial space (mitochondrial matrix). Mitochondrial gene expression is coordinately

regulated through mtDNA and nuclear DNA. The mtDNA is a circular 16,569-base pair (bp) molecule that encodes 13 genes that are involved in oxidative phosphorylation. Two rRNAs and 22 tRNAs are necessary for the expression of mtDNA gene products (Figure 6.5(B)). On the other hand, greater than 99% of all known mitochondrial proteins (~1000 different proteins) are synthesized on cytosolic ribosomes as precursor proteins that must be imported into the organelle. The import of these proteins into mitochondria is performed via a translocase located on the outer membrane and a translocase located on the inner membrane complex (TOM/TIM complex) by the use of presequences, a mitochondrial targeting signal peptide (MTS) (Bohnert, Pfanner, & van der Laan, 2007; Mayer, Neupert, & Lill, 1995; Schatz, 1996). A recent study reported that not only proteins but also noncoding RNAs such as tRNA, rRNA, and microRNA (miRNA) transcribed from nuclear DNA are delivered to mitochondria via the mitochondrial RNA import machinery (Duchene, Pujol, & Marechal-Drouard, 2009; Smirnov, Entelis, Martin, & Tarassov, 2011; Sripada, Tomar, & Singh, 2012).

The vast majority of diseases of mitochondrial origin result from incorporation of damaged or mutated proteins into complexes of the electron transport chain (DiMauro, 2006; Martin, 2006; Sarzi et al., 2007). Any of the 13 genes that are encoded by the mitochondrial genome or those encoded by a nuclear gene may be damaged, as the complexes are composed of subunits encoded by both mitochondrial and nuclear genes. Our focus was on the relationships between mitochondrial diseases and mutations and/or defects in mtDNA. Genetic mutations that are responsible for mtDNA diseases can be categorized into four groups: missense mutations, protein synthesis mutations, deletion mutations, and copy number mutations. Examples of diseases resulting from missense mutations include Leber's hereditary optic neuropathy (LHON) (Newman, 1993) and neurogenic muscle weakness, ataxia, and retinitis pigmentosa (Holt, Harding, Petty, & Morgan-Hughes, 1990). Protein synthesis mutations are in the encoding region of tRNA, which include mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Goto et al., 1990), myoclonic epilepsy with ragged-red fibers (MERRF) (Shoffner et al., 1990), and maternally inherited cardiomyopathy (CM) (Taniike et al., 1992). Deletions in mtDNA have been reported in the majority of chronic progressive external ophthalmoplegia (CPEO) and Kearns–Sayre syndrome cases (Shanske et al., 1990). A marked decrease in the number of mtDNA molecules is associated with familial mitochondrial myopathy (Otsuka et al., 1990).

Since various diseases are caused by mutations in mtDNA, a mitochondrial gene delivery would be expected to serve as a novel strategy for curing a mitochondrial disease. However presently used mitochondrial disease therapies have involved the use of a variety of small molecule drugs (Weissig, Cheng, & D'Souza, 2004), because no useful mitochondrial gene therapeutic technology is currently available. One of the most important functions of mitochondria is the synthesis of ATP, in which various small molecules such as vitamins along with proteins are involved (Saraste, 1999). The dysfunction of this system can be attributed to a substantial number of mitochondrial diseases including MELAS, MERRF, and CPEO (Chinnery & Turnbull, 2000; Quinzii, Hirano, & DiMauro, 2007). To overcome these diseases, coenzymes in the respiratory chain such as vitamin B_1 or B_2, succinic acid and coenzyme Q_{10}, and ATP itself were tested to compensate for mitochondria by oral administration (Nishikawa et al., 1989; Suzuki et al., 1995; Tanaka et al., 1997). Although these strategies resulted in positive therapeutic effects, to some extent, they were not sufficient to permit the loss of mitochondrial function to be completely recovered. Without a drug carrier, the mitochondrial delivery of therapeutic agents in the body could not be actively controlled and, therefore, would be insufficient for therapeutic activity.

In contrast, Koga et al. (2007) reported that an oral administration of L-arginine without a carrier was an effective therapy for MELAS patients. They showed that L-arginine infusions significantly improved all symptoms indicative of a stroke in the acute phase of MELAS, and oral administration significantly decreased the frequency and severity of stroke-like episodes. Moreover, two years of oral supplementation with L-arginine improved endothelial function to the level of controls and normalized plasma L-arginine concentrations in patients. They concluded that L-arginine therapy is promising for the treatment of stroke-like episodes in MELAS. More recently, Sadun et al. evaluated the safety and efficacy of EPI-743 in patients with LHON who were actively losing vision, on an emergency treatment basis. EPI-743 is an experimental therapeutic agent that has been developed by Edison Pharmaceuticals, Inc. (Mountain View, CA, USA) for life-threatening inherited respiratory chain diseases associated with mitochondria (Sadun et al., 2012). In small open-label trial, EPI-743 arrested disease progression and reversed vision loss in all but 1 of 5 consecutively treated patients with LHON. Currently, Phase II clinical trial of the EPI-743 to target patients with LHON are performed in Europe and the United states.

4.2 Mitochondrial Gene Therapeutic Strategies

As discussed above, mitochondrial disease therapies using small molecule drugs result in positive therapeutic effects, to some extent. While these therapeutic strategies are not a curative therapy but, rather, a symptomatic therapy, and require administration for long periods of time. Thus, a mitochondrial gene delivery leading to the complementing normal mtDNA, the repair of mutated mtDNA, inhibition of replication for mutated mtDNA, and/or degradation of mutated mtDNA would be expected to serve as a novel strategy for curing a mitochondrial disease (Yamada & Harashima, 2008). These innovative approaches can be a curative therapy, which permits the loss of mitochondrial function to be completely recovered, and could be achieved by rescuing a defect by the expression of an engineered gene product from the nucleus (allotropic expression), the import of therapeutic agents including wild-type mtDNA into mitochondria (mitochondrial transfection), and other strategies. Kyriakouli, Boesch, Taylor, and Lightowlers (2008) summarize research concerning mitochondrial gene therapy by allotropic expression in an excellent review. Here, we mainly discuss mitochondrial gene therapy using mitochondrial transfection.

4.2.1 Mitochondrial Gene Therapeutic Strategy by Delivery of the Mitochondrial Genome (mtDNA)

In an ideal situation, the mtDNA is completely free of mutations. Cells in this situation are denoted as homoplasmy. However, in certain situations, cells can contain both mutant and wild-type mtDNA. This situation is denoted as heteroplasmy. In the case of mitochondrial related diseases, when the percentage of mutant mtDNA exceeds a certain threshold level, mitochondrial dysfunction becomes clinically apparent (Holt et al., 1990; Kagawa, Inoki, & Endo, 2001). Accordingly, the delivery of a large number of wild-type mtDNA molecules into the mitochondrial matrix in diseased cells would decrease the percent of mutated mtDNA, resulting in the suppression of a mitochondrial disease.

4.2.2 Approach for Mitochondrial Gene Therapy Using Oligodeoxynucleotides and Peptide Nucleic Acid

The loci of mutations in mtDNA have been reported in many mitochondrial diseases. For example, the major point mutations found in MERRF, MELAS, and CM are localized at bp 8344 (A to G; $tRNA^{Lys}$) (Shoffner et al., 1990), bp 3243 (A to G; $tRNA^{Lue\ (UUR)}$) (Goto et al., 1990), and bp 4269 (A to G; $tRNA^{Ile}$) (Taniike et al., 1992), respectively. Accordingly, the precise correction of mutations in mtDNA represents a sophisticated strategy for

genetic disorders. Using a cell-free DNA repair assay with isolated rat liver mitochondria, Chen et al. demonstrated that, not only the nucleus, but also mitochondria contain the machinery required for the repair of genomic mutations using oligodeoxynucleotides (ODN). They also showed that the levels of gene conversion with mitochondrial extracts were similar to those observed with nuclear extracts. Therefore, the delivery of therapeutic ODN to the mitochondrial matrix is promising for the treatment of such mitochondrial diseases (Chen et al., 2001).

Taylor et al. proposed the selective inhibition of mutant mtDNA replication, which would allow the propagation of only wild-type DNA, as treatment for mitochondrial disease (Taylor, Chinnery, Turnbull, & Lightowlers, 1997). Taylor et al. validated this approach by synthesizing peptide nucleic acids (PNAs) complementary to human mtDNA templates containing a deletion breakpoint or a single base mutation to cause a disease. They showed that the antigenomic PNAs specifically inhibited the replication of mutant, but not wild-type mtDNA templates using an in vitro replication run-off assay. They concluded that their antigenomic PNA therapy could help patients with heteroplasmic mtDNA disorders.

4.3 Current Status of Mitochondrial Gene Vectors

As described above, the delivery of therapeutic agents, including nucleic acids such as ODN and PNA, and mtDNA, to the mitochondrial matrix holds promise for the treatment of mitochondrial diseases that result from mtDNA mutations. Here, we discuss the current state of our knowledge concerning the mitochondrial delivery of nucleic acids.

4.3.1 Mitochondrial Delivery of Oligonucleotides Using MTS

To date, it has been reported that DNA is introduced into isolated mitochondria by covalently linking the MTS to either ODN or double-stranded DNA (Seibel et al., 1995; Vestweber & Schatz, 1989). Seibel et al. showed that these conjugates were imported into mitochondria through the outer membrane and the inner membrane via a TOM/TIM complex. The reported length of DNA applicable to this strategy is from 17 bp to 322 bp (Seibel, et al., 1995). A similar strategy has also been developed for the mitochondrial delivery of PNA. With the help of a membrane permeability toxin as a device for cytoplasmic delivery, MTS-conjugated PNA was imported into mitochondria. Their method would be expected to provide a viable strategy for the genetic modification of mitochondria in cultured cells, animals, and patients (Flierl et al., 2003).

4.3.2 Strategy for Delivering Circular DNA Using a Nanocarrier

Polymeric nanoparticles offer an advantage over other materials, because they are easily chemically modified and conjugated to targeting ligands. Lee and coworkers developed MTS-conjugated polyethyleneimine (PEI) for the delivery of DNA to mitochondria (Lee et al., 2007). The localization of the MTS-conjugated PEI/DNA complex to the mitochondrial site was confirmed in living cells. In this strategy, the carrier is internalized into a cell via endocytosis and escapes from endosomes via the proton-sponge effect. Lyer and coworkers also developed an MTD-TFAM technology, in which a recombinant human mitochondrial transcription factor A engineered with an N-terminal PTD followed by the MTS. This technology delivered an mtDNA cargo to mitochondria of living cells (Iyer et al., 2009; Keeney et al., 2009). In this strategy, MTD-TFAM binds mtDNA and rapidly transports it across plasma membranes via transmembrane transportation by PTD. These particles that are complexed with MTS are targeted to mitochondria via the MTS import machinery, however, there are concerns regarding the mitochondrial internalization of the complexed particles because mitochondrial delivery by MTS is severely limited by cargo size (Endo, Nakayama, & Nakai, 1995).

As vesicles for mitochondrial delivery, it has been reported that DQAsomes developed by Weissig and coworkers (Weissig, 2011; Weissig, D'Souza, & Torchilin, 2001), and the DF-MITO-Porter, developed in our laboratory (Yamada, Akita, & Harashima, 2012; Yamada, Furukawa, Yasuzaki, & Harashima, 2011) are both viable vesicles. We discuss our current knowledge regarding DQAsomes, and summarize our efforts to develop a DF-MITO-Porter in the next section. Weissig and coworkers attempted to deliver pDNA to mitochondria using DQAsomes, which are liposome-like vesicles composed of positively charged lipophilic compounds, i.e., dequalinium (DQA) (D'Souza, Rammohan, Cheng, Torchilin, & Weissig, 2003; Weissig et al., 2001). In a previous study, they showed that DQAsomes-DNA complexes (DQAplexes) selectively release pDNA upon contact with isolated mitochondria (Weissig et al., 2001). In addition, DQAplexes apparently escape from endosomes without the loss of their pDNA, and specifically release pDNA proximal to mitochondria in living cells (D'Souza et al., 2003). Intracellular observations showed that MTS-conjugated DNA was developed to further enhance the mitochondrial localization of DNA transfected by DQAsomes (D'Souza, Boddapati, & Weissig, 2005).

4.4 Mitochondrial Delivery of Nucleic Acids Using the DF-MITO-Porter, a Mitochondrial Gene Vector

We developed a nanocarrier for mitochondrial delivery, namely, the DF-MTS-MITO-Porter, to regulate the complicated intracellular multiple processes. As shown in Figure 6.6, cargoes are encapsulated in mitochondria-fusogenic inner lipid envelopes, and coated with endosome-fusogenic outer lipid envelopes. The surface of the DF-MTS-MITO-Porter is modified with a high density of octaarginine (R8), which permits the efficient internalization by cells through macropinocytosis (Khalil, Kogure, Futaki, et al., 2006). Once inside the cell, the carrier escapes from the endosome into the cytosol via membrane fusion, a process that is mediated by the outer endosome-fusogenic envelopes. The carrier then targets mitochondria via the MTS import machinery and fuses with the mitochondrial membrane to deliver the cargos to mitochondria. We are hopeful that the MITO-Porter system can be used to deliver a wide variety of carrier-encapsulated molecules to mitochondria via membrane fusion. Our efforts regarding the development of MITO-Porter are summarized as follows.

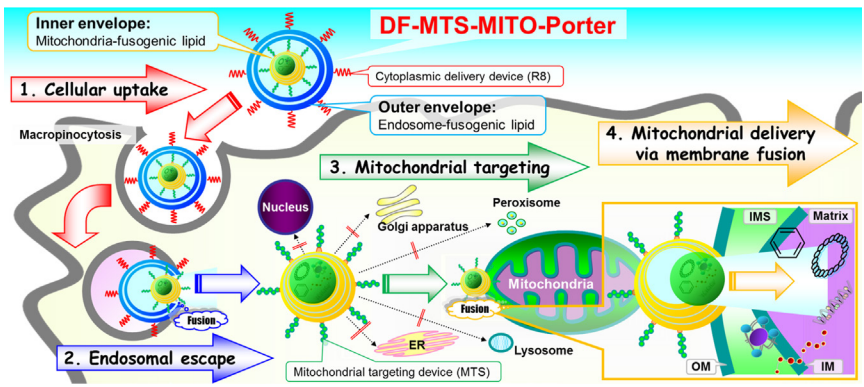

Figure 6.6 ***Schematic image of mitochondrial delivery by the DF-MTS-MITO-Porter.*** Cargoes are encapsulated in a mitochondria-fusogenic lipid envelope (inner), and coated with an endosome-fusogenic lipid envelope (outer). The surface of the DF-MTS-MITO-Porter is modified with a high density of R8, which is efficiently internalized by cells (first step). Once inside the cell, the carrier escapes from the endosome into the cytosol via membrane fusion, a process that is mediated by the outer endosome-fusogenic lipid membranes (second step). The carrier then targets mitochondria via the MTS import machinery (third step) and fuses with the mitochondrial membrane to deliver cargos to mitochondria (fourth step). ER, Endoplasmic reticulum. MTS, mitochondrial targeting signal peptide, OM, outer membrane; IMS, intermembrane space; IM, inner membrane, R8, octaarginine. *This figure is reproduced from Kajimoto, Sato, Nakamura, Yamada, and Harashima (2014).*

4.4.1 Validation of MITO-Porter Concept to Achieve Mitochondrial Delivery via Membrane Fusion

The first barrier to intracellular targeting is the plasma membrane. In a previous study, we showed that high-density R8-modified liposomes are taken up mainly through macropinocytosis and delivered to the cytosol while the aqueous phase marker is retained (Khalil, Kogure, Futaki, et al., 2006). Therefore, we chose R8 as a cytosol delivery device for the MITO-Porter. We also expected that R8, which mimics TAT, might also have mitochondrial targeting activity (Del Gaizo & Payne, 2003; Del Gaizo, MacKenzie, & Payne, 2003). To achieve mitochondrial delivery via membrane fusion based on the MITO-Porter concept, we screened for fusogenic activities directed at isolated mitochondria by monitoring the cancellation of FRET (Yamada et al., 2008).

R8-modified liposomes (R8-LP) composed of DOPE showed a higher fusogenic activity than liposomes comprised of egg yolk phosphatidylcholine (EPC). Fusogenic activity was inadequate in the absence of R8. These results suggest that strong electrostatic binding between R8-LP and mitochondria stimulates liposomal fusogenic activity. Two highly fusogenic lipid compositions that were identified during the screening were used in constructing the MITO-Porter. These were R8-LP composed of DOPE and contains either sphingomyelin (SM) or PA (Figure 6.7(A)). Moreover, lipid compositions containing SM had a lower cytotoxicity than those containing PA, indicating that the lipid composition, DOPE/SM/R8 (9:2:1), was optimal for the mitochondrial delivery of the carriers (Yamada et al., 2011).

Using the GFP as a model macromolecule and fluorescence imaging of the intracellular trafficking, we were able to confirm that the MITO-Porter is, in fact, capable of delivering a macromolecule to mitochondria (Figure 6.7(B)) (Yamada et al., 2008). Additionally, transmission electron microscopy (TEM) analyses showed that gold colloid as a model macromolecule could be delivered by the MITO-Porter to the interior of mitochondria (Figure 6.7(B)) (Yamada et al., 2008). Moreover, we were able to verify that the MITO-Porter delivered cargoes to the mitochondrial matrix, which contains mtDNA pools, using propidium iodide (PI), as a probe to detect mtDNA (Yasuzaki, Yamada, & Harashima, 2010). In a previous paper, we confirmed that this system can be used to efficiently visualize mtDNA, not only in isolated mitochondria, but in living cells as well. Based on our previous report, we conclude that the MITO-Porter has the ability to deliver cargoes to the mitochondrial matrix.

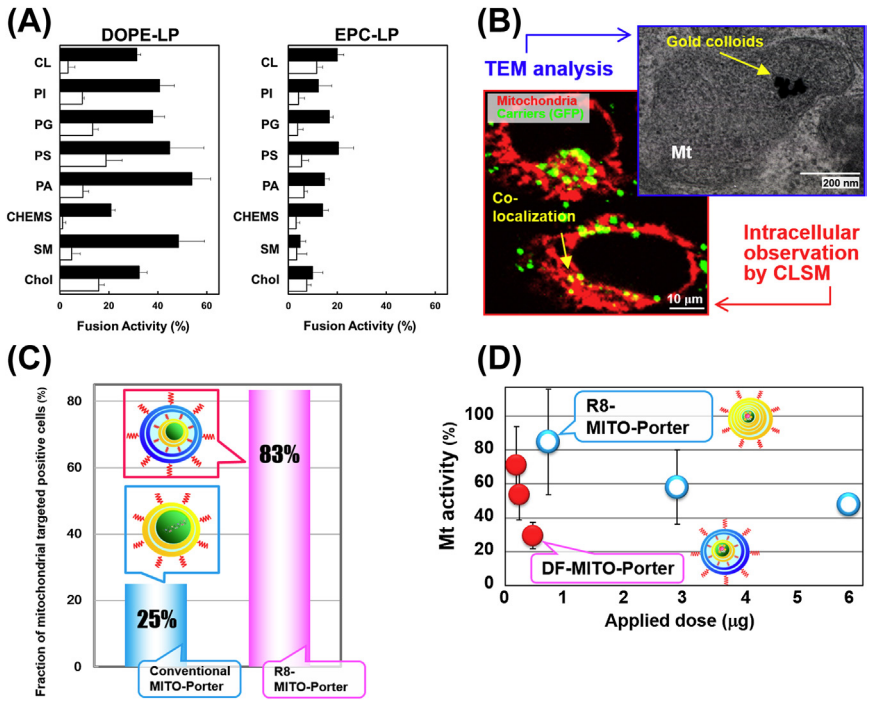

Figure 6.7 ***Summary of experimental results concerning mitochondrial delivery by MITO-Porter system.*** Screening of fusogenic lipid compositions with the mitochondrial membrane (A). Fusion activities (%) of DOPE-LP and EPC-LP were calculated in terms of the reduction of FRET. Closed bars, R8-LP; open bars, unmodified liposome. Data are represented by the mean ± S.D. ($n = 3$). Intracellular observation of the MITO-Porter (green) after staining mitochondria (red) and TEM analysis indicating that Gold colloids encapsulated in the MITO-Porter were delivered to mitochondria (B). The fraction of mitochondrial targeted positive cells was calculated using a confocal image-assisted integrated quantification method (C). Comparison of mitochondrial activity after DNase I delivery between DF-MITO-Porter and R8-MITO-Porter (D). The closed and open circles represent the values corresponding to the mitochondrial activity (%), when the DF-MITO-Porter and the R8-MITO-Porter were used. Data are represented as the mean ± S.D. ($n = 3$–4). (See the color plate.) *These figures are reproduced from Nakamura et al. (2012), Yamada et al. (2008), Yamada et al. (2011).*

4.4.2 Construction of DF-MITO-Porter and Mitochondrial Delivery of Nucleic Acids

To construct a higher performing MITO-Porter, the MITO-Porter was coated with endosome-fusogenic outer envelopes to produce the DF-MITO-Porter. It was expected that the DF-MITO-Porter would efficiently deliver cargoes to mitochondria, through the endosomal and mitochondrial membranes via

stepwise membrane fusion (Figure 6.6). Intracellular observations showed that the DF-MITO-Porter delivered exogenous macromolecules into mitochondria in living cells more effectively than conventional R8-MITO-Porter (Yamada, Akita, et al., 2012; Yamada et al., 2011) (Figure 6.7(C)).

Furthermore, we attempted the mitochondrial delivery of DNase I protein using the DF-MITO-Porter to estimate the mitochondrial gene targeting of the carrier (Yamada, Akita, et al., 2012; Yamada et al., 2011; Yamada & Harashima, 2012). It was expected that mtDNA would be digested, followed by a decrease in mitochondrial activity, as the mitochondrial matrix delivery of DNase I progressed. Figure 6.7(D) provides information on the applied dose of DNase I (x-axis) and mitochondrial activity (y-axis). We calculated the effective dose 50 (ED_{50}) for each carrier, and the results indicated that the DF-MITO-Porter ($ED_{50} = 0.33\,\mu g$) was 15-fold more efficient than the R8-MITO-Porter ($ED_{50} = 5.4\,\mu g$) for mitochondrial delivery (Yamada & Harashima, 2012). We also evaluated mtDNA-levels within the cells after the delivery of the DNase I protein using PCR, followed by EtBr staining after separation by electrophoresis (Yamada et al., 2011; Yamada & Harashima, 2012). The results indicated that the use of the DF-MITO-Porter resulted in a decrease in mtDNA-levels, whereas the carriers with low fusion characteristics had only a negligible effect on mtDNA-levels. The results provided a demonstration of its potential for use in therapies aimed at mtDNA.

To demonstrate that the MITO-Porter is a useful mitochondrial vector for nucleic acids, we attempted the mitochondrial delivery of nuclei acids using the MITO-Porter system (Yamada, Kawamura, & Harashima, 2012). We succeeded in packaging an oligonucleotide in the DF-MITO-Porter. Cellular uptake analysis using flow cytometry showed that DF-MITO-Porter efficiency internalized oligo DNA into the cells. Intracellular observations showed that the DF-MITO-Porter was effectively released from endosomes. Moreover, the findings confirmed that the mitochondrial targeting activity of the DF-MITO-Porter was significantly higher than that of a carrier without outer endosome-fusogenic envelopes, namely, the R8-MITO-Porter. These results support the conclusion that mitochondrial-targeted DNA delivery using a DF-MITO-Porter can be achieved when intracellular trafficking is optimally regulated.

4.4.3 Integration of the DF-MITO-Porter with Mitochondrial Targeting Signal Tags

Our previous analysis of intracellular trafficking indicated that DF-MITO-Porter improved the endosomal escape process of the R8-MITO-Porter, while the rate-limiting step in mitochondrial delivery using the carriers

appears to be the mitochondrial targeting process from the cytosol (Yamada, Kawamura, et al., 2012). A previous investigation using isolated mitochondria showed that the mitochondrial binding activity of liposomes increased significantly after modification with R8, a cationic peptide (Yamada & Harashima, 2008), probably because of a high-negative potential of mitochondria. Unfortunately, the R8-modified inner envelope in the DF-MITO-Porter failed to sufficiently control the mitochondrial targeting process in living cells, as opposed to that for isolated mitochondria. It was recently reported that the conjugation of MTS conferred selective mitochondrial delivery, not via electrostatic interaction (Flierl et al., 2003; Schatz, 1996), but this strategy failed in cases of macromolecules and hydrophobic molecules (Endo et al., 1995; Esaki, Kanamori, Nishikawa, & Endo, 1999; Gruhler, Ono, Guiard, Neupert, & Stuart, 1995). Moreover, this strategy should include cytoplasmic delivery, because MTS itself cannot be internalized through the cellular membrane.

Thus, we attempted to integrate a DF-MITO-Porter system with an MTS to achieve the effective mitochondrial delivery in living cells as shown in Figure 6.6. We first synthesized a lipid derivative that is conjugated with an MTS, and constructed an MTS-MITO-Porter. We then evaluated the mitochondrial targeting activity of the MTS-MITO-Porter in a cell homogenate where other organelles are present, in addition to mitochondria. The results indicated that the MTS-MITO-Porter targeted mitochondria more efficiently than the R8-MITO-Porter (Yamada & Harashima, 2013) (Figure 6.8(A)). We also investigated the mitochondrial membrane fusion activity of the MTS-MITO-Porter. The results indicate that the MTS-MITO-Porters and R8-MITO-Porters had a significantly higher fusogenic activity than nonmodified liposomes (Figure 6.8(B)). The results indicate that a combination of MTS and the MITO-Porter would be useful for selective mitochondrial delivery via membrane fusion.

We also coated the MTS-MITO-Porter with outer R8-modified envelopes to construct a DF-MTS-MITO-Porter. Intracellular observations showed that the mitochondrial targeting of the DF-MTS-MITO-Porter was enhanced compared with that of DF-R8-MITO-Porter (Kawamura, Yamada, Yasuzaki, Hyodo, & Harashima, 2013) (Figure 6.8(C)). More recently, we evaluated the utility of mitochondrial targeting functional peptides as a ligand for delivering carriers using isolated mitochondria, homogenate, and living cells. The S2-peptide (Dmt-$_D$-Arg-FK-Dmt-$_D$-Arg-FK-NH_2)-modified DF-MITO-Porter (DF-S2-MITO-Porter) showed a high mitochondrial targeting activity in both homogenates and living cells.

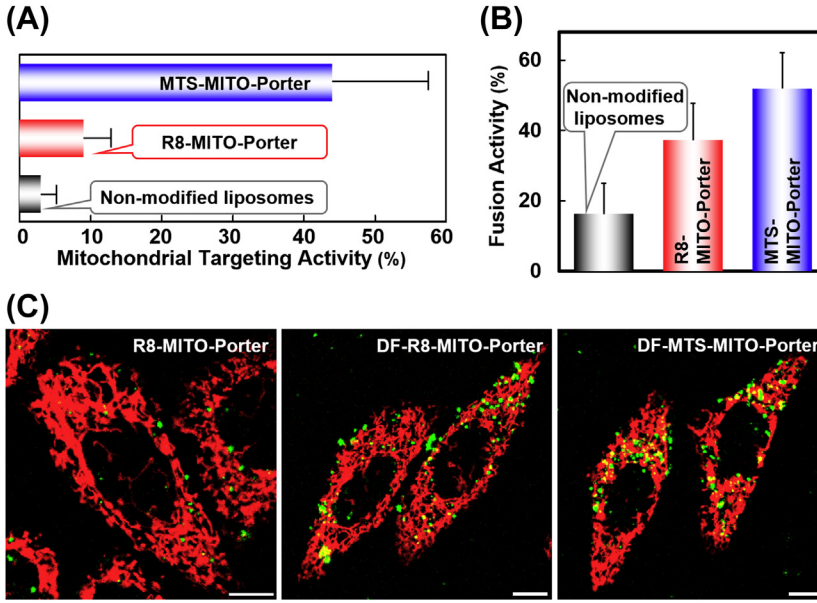

Figure 6.8 ***Evaluation of mitochondrial targeting of MITO-Porter integrated with MTS.*** Mitochondrial targeting activity of the MTS-modified MITO-Porter, R8-modified MITO-Porter, and nonmodified liposomes (A). Mitochondrial membrane fusion activity of the MTS-MITO-Porter, the R8-MITO-Porter, and nonmodified liposomes (B). Data are the means ± S.D. ($n = 3–4$). Intracellular observation of carriers (R8-MITO-Porter, DF-R8-MITO-Porter, DF-MTS-MITO-Porter) (C). The carrier (green) was observed to co-localize with red stained mitochondria, observed as a yellow signal. Scale bars, 10 μm. (See the color plate.) *These figures are reproduced from Kawamura et al. (2013), Yamada and Harashima (2013).*

In addition, the S2 peptide possessed a low cellular toxicity compared to R8 while mitochondrial targeting activity in homogenate solution and living cells were similar to that of R8. The findings reported herein indicate that the S2 peptide can be an ideal modifier for a mitochondrial targeting nanocarrier.

4.5 Perspective Regarding Mitochondrial Gene Vectors

Mitochondria were recognized as the energy plant in a cell and came into the limelight as the organelle that regulates apoptosis. Currently, numerous relationships between mitochondrial dysfunction caused by mtDNA mutations and various diseases have been reported. Therefore, mitochondrial gene therapy and diagnosis would be expected to have substantial medical benefits. In this section, issues related to mitochondrial diseases and

mitochondrial gene therapeutic strategies are discussed. We also summarize current reports concerning the mitochondrial delivery of nucleic acids. We hope that these mitochondrial vectors will contribute to successful mitochondrial therapies.

Our findings indicate that the DF-MITO-Porter holds promise as a mitochondrial gene vector. We are currently attempting to control mitochondrial gene expression using an MITO-Porter system. Our ultimate goal is to achieve successful mitochondrial therapy using a mitochondrial DDS. To reach this ultimate goal, we believe that mitochondrial DDS requires collaboration with experts in various fields of research (e.g., genetics, molecular biology, pathology, and so on), to develop a strong proof of concept for mitochondrial gene therapy. The concept and establishing technology should then be put to practical use through collaboration with industry, government, and academic institutions. It is our hope that these optimized delivery systems will provide effective therapies for the many patients who suffer from mitochondrial diseases.

5. YSK-MEND

5.1 siRNA Delivery to Hepatocytes In vivo

5.1.1 Currently Developed Carriers

Short interfering RNA (siRNA) delivery to liver tissue is important in the treatment of over 4000 human diseases caused by genetic liver disorders (McClellan & King, 2010) and infectious diseases such as hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. As is known, almost all of these diseases originate from hepatocytes (Krawczyk, Müllenbach, Weber, Zimmer, & Lammert, 2010). The development of an efficient and safe siRNA delivery carrier to hepatocytes is one of the most important means to target genes that are not amenable to treatment with a drug and to treat a variety of intractable diseases. However, although numerous carriers have been reported, only several have succeeded in sufficient silencing of hepatocyte genes in vivo. For example, an ionizable lysine-based lipid containing liposomes induced a higher gene silencing activity than lipofectamine 2000 in *in vitro* cultured HeLa cells expressing luciferase, but failed to silence coagulation factor VII (FVII), a hepatocyte-specific gene, in mice at a dose of 5 mg/kg (Walsh, Nguyen, Tiffany, & Szoka, 2013). Lipid nanoparticles containing a cationic lipid composed of tris(2-aminoethyl) amine and linoleyl chains induced only a 30% FVII gene silencing at a dose of 1 mg/kg (Yu et al., 2012). Marina Biotech synthesized a cationic

lipid, DiLA2, and successfully induced FVII gene silencing (Adami et al., 2011). In this system, a pH-sensitive anionic lipid, CHEMS restrained the cationic property of DiLA2 in the blood stream and played a significant role in gene silencing activity. The most advanced siRNA delivery carrier to hepatocytes is the stable nucleic acid lipid particle (SNALP). It was first reported that the first generation SNALP containing ionizable amino lipid, DLin-DMA induced apolipoprotein B (ApoB) gene silencing in nonhuman primates (Zimmermann et al., 2006). Subsequently, through a rational design and investigations of structure–activity relationships of ionizable amino lipids, DLin-KC2-DMA and DLin-MC3-DMA were developed and the second generation MC3-SNALP achieved a 50% FVII gene silencing at a dose of 0.005 mg/kg in mice (Jayaraman et al., 2012; Semple et al., 2010). This carrier is currently in clinical trials for the treatment of transthyretin-mediated amyloidosis and hypercholesterolemia. Another promising system is the Dynamic PolyConjugate. The current strategy of this system involves the simple co-injection of a hepatocyte-targeted *N*-acetylgalactosamine (GalNAc)-conjugated mellitin-like peptide and cholesterol-conjugated siRNA. This system was well tolerated and achieved a 50% FVII gene silencing at a dose of 0.01 mg/kg in mice, similar level to MC3-SNALP (Wooddell et al., 2013). This system is also in a clinical trial for the treatment of HBV infection. The breakthrough in the efficacy of siRNA delivery resulted in the expansion of therapeutic window and it is anticipated that RNAi medicine will be realized in the near future.

5.1.2 Development of YSK05-MEND

Until now, numerous cationic lipids have been developed for improving the transfection efficiency of functional nucleic acids such as plasmid DNA and siRNA (Zhi et al., 2013). We also recently developed an original cationic lipid, YSK05 (Sato, Hatakeyama, Hyodo, Akita, & Harashima, 2012; Sato, Hatakeyama, Sakurai, et al., 2012). YSK05 is composed of a tertiary amino group, a ketal linker, and two long unsaturated carbon chains. The tertiary amino group can be protonated in an acidic environment such as endosomal/lysosomal compartment but not at physiological pH such as in the blood stream. The ketal linker provides metabolic stability against esterases and prevents inactivation of the MEND before cytosolic siRNA delivery. The two long unsaturated carbon chains emphasize cone shape and facilitates fusion with biomembrane through phase transition from lamellar phase to inverted hexagonal phase. Taken together, the MEND containing YSK05 (YSK05-MEND) converts from near-neutral to a cationic

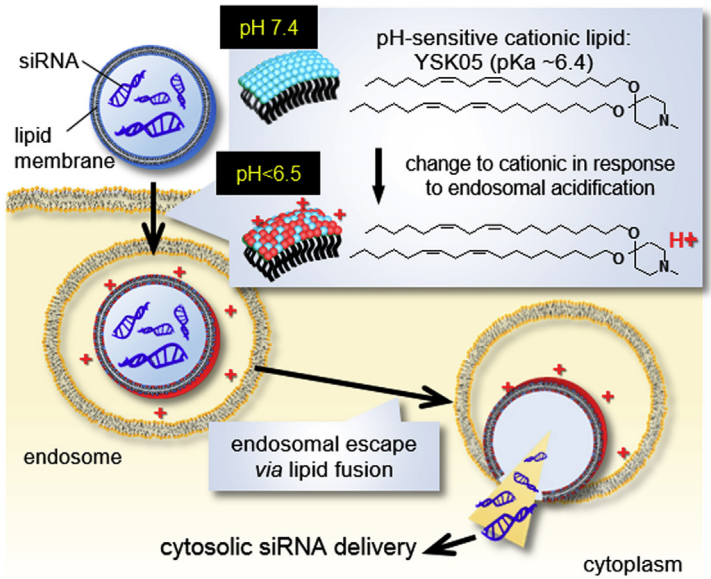

Figure 6.9 ***Schematic representation of the process of cytosolic siRNA delivery by the YSK05-MEND.*** The YSK05-MEND is taken up into cell by endocytosis. The YSK05 is protonated in response to endosomal acidification, which promotes the release of siRNA into cytosol via fusion with endosomal membranes.

molecule in response to endosomal acidification and efficiently releases siRNA to the cytoplasm via membrane fusion (Figure 6.9). The YSK05-MEND successfully induces a higher gene silencing activity compared to MENDs containing conventional cationic lipids such as 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) or DODAP, and to a commercially available transfection agent, Lipofectamine 2000 in in vitro cultured HeLa cells expressing dual luciferase. We also confirmed that the acidification of endosomes is an essential step for the cytosolic siRNA delivery by the YSK05-MEND using endosome buffering agents, such as chloroquine and ammonium chloride. Moreover, we found that a helper lipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE) resulted in a higher efficacy of cytosolic siRNA delivery compared to various phosphatidylcholines and DOPE, which is used for providing fusogenicity to lipid based carriers. It was reported that the stability of the siRNA carrier in serum and the intracellular environment would be needed for efficient cytosolic delivery (Alabi et al., 2013), and the POPE containing YSK05-MEND consistently protected the encapsulated siRNA compared to a DOPE containing YSK05-MEND.

5.1.3 Optimization of YSK05-MEND for Hepatocytes

A complement protein C3 covalently binds to phosphatidylethanolamine through a C3 thioester bond (Mold, 1989). It is also known that the C3 can recognize primary and secondary amino groups but not tertiary amino groups, the structure of the cationic lipid should be carefully designed so as not to activate the alternative complement pathway. Since YSK05 contains no functional groups that can react with the C3 protein, we concluded that YSK05 could be used for in vivo applications. Based on the above information, we modified the lipid composition of the YSK05-MEND to apply it for hepatocytes in vivo. First, POPE was excluded from the lipid composition in order to prevent the activation of the complementary pathway and subsequent recognition and elimination from the blood stream by the reticuloendothelial system. The lipid composition of YSK05-MEND was then optimized based on the scavenger receptor type B class I (srbI) gene in the liver and the optimized formulation composed of YSK05/Chol/PEG-DMG at molar ratios of 70/30/3% achieved a 50% FVII gene silencing at a dose of 0.06 mg/kg in mice (Watanabe et al., 2014). We also found that the ratio of YSK05/cholesterol in the lipid composition significantly affected gene silencing activity in liver tissue. Specifically, the activity dramatically increased with increasing YSK05 until 70 mol% was reached. This effect can be explained by the increased efficiency of endosomal escape, because the hemolytic activity of YSK05-MEND significantly increased through the optimization of lipid composition. On the other hand, gene silencing activity gradually decreased as with increasing YSK05 up to 80 mol%. This can be explained by the fact that lipid compositions with less than 20 mol% cholesterol are unstable in the blood stream (Schroeder, Levins, Cortez, Langer, & Anderson, 2010), resulting in inefficient delivery of siRNA to hepatocytes.

5.1.4 Key Properties for Hepatocyte-specific Delivery

The acid dissociation constant (pKa) value of a nanocarrier surface is critical for its pharmacokinetics, biodistribution, and intracellular trafficking. It was reported that the optimal range of the pKa value of ionizable lipid nanoparticles (iLNP) for hepatocytes was from 6.2 to 6.5 (Jayaraman et al., 2012). The optimized YSK05-MEND for the liver has a pKa value of 6.4, meaning that, theoretically, only about 9% of the YSK05 molecule on the particle surface are protonated (in a cationic form), and typically indicates a near-neutral zeta-potential (0–8 mV) at pH7.4. This neutral surface property prevents electrostatic, nonspecific interaction with serum components and is important for binding with apolipoprotein E (ApoE) in the blood stream.

It was reported that neutral liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine/cholesterol are taken up by hepatocytes and are rapidly eliminated from the blood stream in wild-type mice but circulated for long periods in ApoE knockout mice (Yan et al., 2005). It was reported that the uptake of iLNP by cells largely depends on the existence of ApoE both in vitro and in vivo (Akinc et al., 2010). The optimized YSK-MEND is also taken up by hepatocytes through the interactions with ApoE but it becomes localized, not only in hepatocytes, but also in liver sinusoidal endothelial cells (LSECs) and Kupffer cells. However, an YSK05-derivative with a pKa value lower than 6.0 achieved hepatocyte-specific distribution (unpublished data). These findings indicate that a pKa value of around 6.4 is optimal for gene silencing in hepatocytes but is not optimal in the light of hepatocyte specificity. Actually, the gene silencing of CD31, an endothelial cell marker, can be observed after intravenous administration of the optimized YSK05-MEND encapsulating siCD31 (unpublished data). Although the role of Kupffer cells in the elimination of waste substances and in inflammatory and immune responses has been extensively investigated over the years, the function of LSECs has not been explicitly clarified. Recently, however, the role of LSECs in the clearance of waste products and in immunity has become better understood (Sørensen et al., 2012). It was also reported that cationic liposomes can induce apoptosis in macrophages through the generation of reactive oxygen species and the activation of the p38 MAPK cascade in vitro (Iwaoka, Nakamura, Takano, Tsuchiya, & Aramaki, 2006). Moreover, it was reported that a high dose of an ionizable cationic lipid, CLin-DMA, resulted in inflammation and cytokine production through the ERK1/2 MAPK cascade in mice (Abrams et al., 2010). Therefore, the localization of a nanocarrier for LSECs and Kupffer cells should be minimized to prevent cationic liposome-mediated inflammation and the siRNA-mediated stimulation of innate immunity via the TLR3.

To achieve hepatocyte-specific localization, both minimizing recognition by scavenger cells and active targeting to hepatocytes are important issues. PEG modification has been widely adopted to prevent interactions with biocomponents and confer stealth to nanoparticles and to extend the circulation time. At the same time, PEG modification leads to the stabilization of the nanocarrier, resulting in a dramatic decline in both cellular uptake for target cells and membrane fusogenicity with the endosomal membrane. We refer to this phenomenon as the PEG-dilemma (Hatakeyama, Akita, & Harashima, 2013). To overcome this problem, careful adjustment of the amount of PEG modification and/or the specific strategy to avoid the

dilemma is needed. The modification of a specific ligand on a nanoparticle surface is a widely used method for achieving specific targeting. To target hepatocytes, galactose (Gal) and GalNAc are mainly used. Gal and GalNAc specifically bind to the asialoglycoprotein receptor (ASGPR) which is specifically expressed on the surface of hepatocytes. Although the affinity of GalNAc for ASGPR is higher than Gal, the dissociation constant (Kd) value is too high (in the micro molar level) to achieve sufficient affinity. It is known that multivalent interactions between ligand-PEG conjugations on the surface of nanoparticles and receptors on the cell surface results in an enhanced affinity (Takara et al., 2012). The effect of clustered ligands has been investigated to further enhance ligand affinity (Sliedregt et al., 1999). Specifically, it has been reported that a trivalent GalNAc ligand in which the distance between each GalNAc molecule is around 2 nm has the appropriate disposition for achieving a high affinity to ASGPR. It was recently reported that the mechanism responsible for the uptake of iLNP by hepatocytes could be clearly changed from ApoE/LDLR-mediated to ASGPR-mediated by modification of the surface with a multivalent GalNAc-PEG conjugate (Akinc et al., 2010).

Controlling size of the nanoparticles is very important for the efficient delivery of siRNA to hepatocytes, in that elimination by phagocytes can be eliminated and the efficiency of passing through fenestrae from the blood to space of Disse can be maximized. Also, it is known that the diameter of the fenestrae is critical for the efficiency of transgene expression by the adenoviral gene vector (Jacobs, Wisse, & De Geest, 2010). Therefore, it can be estimated that a formulation with a small diameter and monodispersed size distribution is ideal for targeting hepatocytes. In the formulation process used in preparing a carrier, it is important to control particle size and other physical properties such as the efficiency of siRNA encapsulation. We examined various formulation protocols and developed the tertiary butanol dilution method (Sato, Hatakeyama, Sakurai, et al., 2012). In this method, only a small amount of siRNA (typically, double digit microgram) is needed for formulation, about 90% of the siRNA can be recovered in an encapsulated form, and particles with a monodispersed size distribution can be obtained. The diameter of the optimized YSK05-MEND for hepatocytes was controlled to around 50 nm based on dynamic light scattering measurements (number mean) and the typical polydispersity was less than 0.2. As it is reported that the average diameter of fenestrae in mice is 141 nm (Wisse, Jacobs, Topal, Frederik, & De Geest, 2008), the size of the optimized YSK05-MEND is sufficient to target mice hepatocytes. However, as it is

also known that the average size of fenestrae in humans is significantly smaller (107 nm) than mice and the density of fenestrae can be reduced in diseases such as hepatitis and hepatic cirrhosis (Warren et al., 2007), the optimal size distribution for clinical applications must be adjusted to compensate for various diseases. We also found that the YSK05-MEND that was 30 nm in diameter showed a significantly lower gene silencing activity compared to a counterpart with a 50 nm diameter (unpublished data). There may be the optimal size for each formulation in terms of achieving maximal gene silencing activity. The cause of the reduced activity in small formulation is currently being investigated.

5.1.5 Application of YSK05-MEND for In vivo Gene Analysis and Therapy

The discovery of siRNA enabled us to examine the function of genes of interest in in vitro cultured mammalian cells. However, gene function in an in vivo environment, which involves complicated interactions with surrounding cells and other various components, would be different compared to an in vitro environment in some degree. Therefore, the development of an siRNA delivery carrier to hepatocytes would permit a deep understanding of the actual function of hepatic genes in an in vivo environment. We recently succeeded in discovering a new promising therapeutic target gene for metabolic diseases by a combination of comparative transcriptome analysis in the liver between diabetic model, kkAy mice and normal control mice and an efficient siRNA delivery carrier to hepatocytes, YSK05-MEND (Figure 6.10(A)) (Hayashi et al., 2014). First, we determined that the expression level of monoacylglycerol O-acyltransferase 1 (Mogat1) in disease model mice was significantly higher compared to healthy control mice. We then found that the repeated injection of the YSK05-MEND encapsulating siMogat1 resulted in significantly improved blood glucose levels, lower serum insulin levels, higher serum adiponectin levels without any obvious toxicity, suggesting that hepatic Mogat1 silencing has the essential effect of preventing type 2 diabetes. Thus, we were able to identify the hepatic Mogat1 gene as a promising therapeutic target gene for type 2 diabetes by direct analysis of the therapeutic potency of Mogat1 silencing in disease model mice.

Furthermore, we demonstrated that the optimized YSK05-MEND formulation can also deliver an anti-microRNA oligonucleotide (AMO) against microRNA-122 to hepatocytes (Figure 6.10(B)). Significant

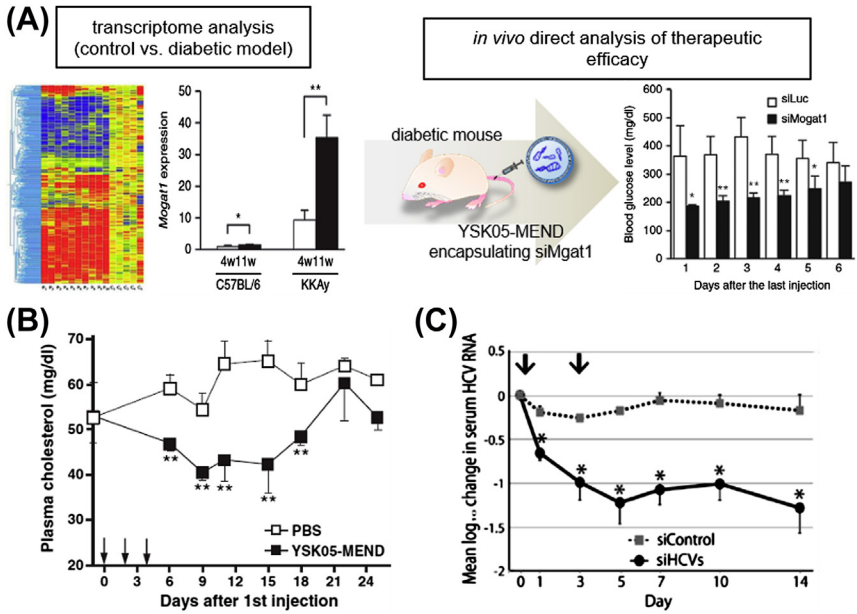

Figure 6.10 ***Various applications of the YSK05-MEND.*** (A) Rapid discovery of a potent therapeutic target gene, Mgat1, by the combination of transcriptome analysis and in vivo siRNA delivery to hepatocytes. Transcriptome analysis revealed an elevated expression of the hepatic Mgat1 gene in diabetic mice. The therapeutic efficacy of hepatic Mgat1 silencing in diabetic mice were evaluated by administration of the YSK05-MEND encapsulating siMgat1. Significant silencing of hepatic Mgat1 gene expression resulted in improved blood glucose level and other therapeutic responses (Hayashi et al. 2014). (B) Administration of YSK05-MEND encapsulating anti-miR122 oligonucleotide resulted in reduction of plasma cholesterol level (Hatakeyama et al., 2014). (C) Administration of YSK05-MEND encapsulating anti-HCV siRNA (siHCVs) into HCV infected mice resulted in reduced level of serum HCV RNA (Watanabe et al., 2014).

de-repression of microRNA-122 target genes such as AldoA, Bckdk, and Ndrg3, and reduced total plasma cholesterol levels were observed after the administration of AMO122 formulated in the YSK05-MEND but not free AMO122 (Hatakeyama et al., 2014; Takahashi et al., 2013). It is known that the repression of microRNA-122 results in the inhibition of HCV replication (Lanford et al., 2010). Separately, the YSK05-MEND formulating anti-HCV siRNAs succeeded in an efficient and long lasting inhibition of HCV in chimeric mice with humanized livers (Figure 6.10(C)) (Watanabe, et al., 2014). Therefore, it is expected that our delivery system can significantly repress HCV through the simultaneous inhibition of HCV genomic RNA by siRNAs and host RNA by an antisense oligonucleotide.

5.2 Solid Tumor

5.2.1 Currently Developed Carriers

Macromolecules that circulate for a long time in the blood stream can passively accumulate in solid tumor tissue. Such passive accumulation results from an abnormal vasculature, which involves an aberrant blood vessel architecture and immature lymphatic drainage, in tumor tissue. We refer to this phenomenon as the enhanced permeability and retention (EPR) effect (Matsumura & Maeda, 1986). Until now, many carriers have successfully delivered siRNA to tumor tissue and induced gene silencing. For example, cyclodextrin-polymer-based nanoparticles, modified with transferrin for targeting transferrin receptor expressing tumor cells, induced gene silencing without any measured innate immune response in a xenograft model for Ewing's sarcoma (Hu-Lieskovan, Heidel, Bartlett, Davis, & Triche, 2005). This carrier entered clinical trials for the treatment of cancer as the first actively targeted siRNA delivery nanoparticle, and successfully induced target gene silencing via an RNAi-mediated mechanism in human melanoma patients (Davis et al., 2010). SNALP also induced gene silencing in hepatic and subcutaneous tumor model mice (Judge et al., 2009). This carrier encapsulating an anti-vascular endothelial growth factor and anti-kinesin spindle protein siRNAs entered clinical trials for tumor patients with hepatic metastasis (Tabernero et al., 2013).

5.2.2 Advantage of the YSK05-MEND for Tumor Targeting

Regarding tumor targeting via the EPR effect, the circulation properties of a nanoparticle in the blood stream is significant factor for effective siRNA delivery (Ozpolat, Sood, & Lopez-Berestein, 2010). We confirmed that the improved circulation time of the YSK05-MEND resulting from PEG modification (PEGylation) resulted in a significantly enhanced siRNA concentration and target gene silencing in tumor tissue (Sakurai et al., 2013). Although PEGylation typically causes a dramatic decrease in the efficiency of intracellular trafficking such as endosomal escape as described above, the YSK05-MEND circumvented the PEG-dilemma to some degree because of the high fusogenic property and pH-sensitivity of YSK05 (Sato, Hatakeyama, Sakurai, et al., 2012). As the YSK05-MEND has a near-neutral charge in the blood stream, a small amount of PEGylation is sufficient to prevent nonspecific electrostatic interactions with serum components. Specifically, only a 5 mol% of PEGylation resulted in the saturation of serum concentration after the intravenous administration of the YSK05 liposomes, whereas 10 mol% or more PEG-lipid was necessary to achieve a

similar serum level in permanently cationic DOTAP containing liposomes (Sakurai et al., 2013). Moreover, once internalized into cells via endocytosis, the YSK05 is immediately converted from the neutral form to a highly cationic form, and the small amount of PEG become insufficient to prevent electrostatic interactions and fusion with the endosomal membrane.

5.2.3 Important Properties for Homogenous Delivery to Tumor Cells In vivo

It has been recognized that the pore size between vascular endothelial cells in tumor tissue is 100 nm or larger in a mice model (Yuan et al., 1995). Therefore, a nanoparticle with a size of around 100 nm can accumulate in tumor tissue via the EPR effect. However, the pore size varies depending on the tumor type and the surrounding microenvironment around the tumor tissue. For example, the cut-off size, which is the largest particle that can penetrate a blood vessel wall from the blood stream to tumor tissue, in subcutaneous tumor tissue is significantly larger than that for cranial tumor tissue (Hobbs et al., 1998). Furthermore, it has recently been hypothesized that, if the EPR effect functions in human patients, because Food and Drug Administration-approved nanomedicines such as Doxil/Caelyx, Daunoxome and Abraxane, would only modestly improve the overall survival of human patients (Jain & Stylianopoulos, 2010), in spite of the fact that these nanomedicines would be expected to have a significantly higher therapeutic potency than their free drugs in some mice models. As the rate of tumor growth in a mouse model is faster than that in human patients, an abnormality of the tumor blood vessel wall and permeability in tumor tissue would be far different. It was recently reported that only micelle with a diameter of 30 nm could penetrate poorly permeable pancreatic tumor tissue, whereas larger micelles failed (Cabral et al., 2011). Also, we found that gene silencing efficiency in tumor tissue was saturated at approximately 50% after an intravenous injection of the YSK05-MEND with a particle diameter of 100 nm at a dose of 3 mg/kg or more (Sakurai et al., 2013). Similar to this, several liposomal siRNA carriers failed to achieve more than 90% gene silencing in tumor tissue (Gao et al., 2011; Hu-Lieskovan, et al., 2005; Judge et al., 2009; Sakurai et al., 2011), unlike in liver. These results can be explained by heterogeneous distribution of the nanocarriers in tumor tissue, resulting from the physicochemical properties of nanoparticles and various tumor microenvironments (Figure 6.11).

Many reports have suggested that tumor stromal component, collagen and hyaluronan, prevent the diffusion of nanoparticles and antibodies

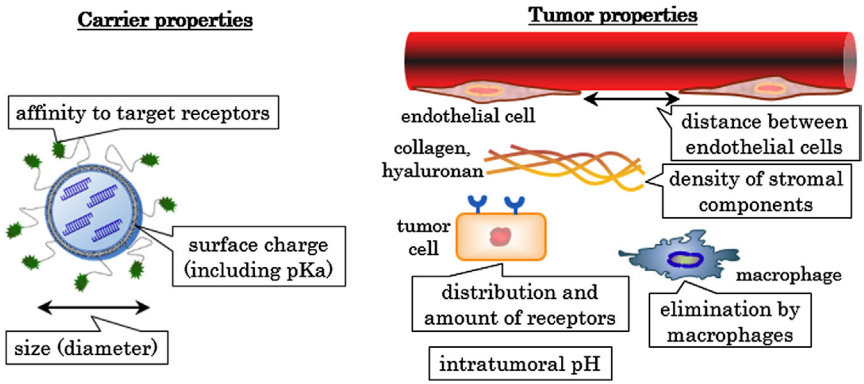

Figure 6.11 ***Various properties affecting the permeability of nanocarriers in tumor tissue.***

(Eikenes, Tari, Tufto, Bruland, & de Lange Davies, 2005; Netti, Berk, Swartz, Grodzinsky, & Jain, 2000; Whatcott, Han, Posner, Hostetter, & Von Hoff, 2011). It is also known that the physicochemical properties of nanoparticles such as particle size, surface charge, and affinity for the target receptor restrict their permeability in tumor tissue. Many researchers have reported on the existence of size dependency on tumor penetration by using various types of nanoparticles including block copolymer micelles, gold nanoparticles, fluorescent nanoparticles (Cabral et al., 2011; Chauhan et al., 2012; Huo et al., 2013; Lee, Fonge, Hoang, Reilly, & Allen, 2010). We also found that a 35-nm-diameter YSK05-MEND could diffuse homogenously in tumor tissues generated by the subcutaneous inoculation of several tumor cells such as human lung adenocarcinoma cells and human colorectal carcinoma cells, while an 80-nm-diameter YSK05-MEND failed to diffuse (unpublished data). Based on these reports, it is clear that controlling the diameter of a nanoparticle to 30 nm or smaller is an important issue in terms of achieving homogeneous drug delivery. Surface charge is also important. It has been reported that both negatively charged and positively charged liposomes bind to components of extracellular matrix and are significantly immobilized (Lieleg, Baumgärtel, & Bausch, 2009). Therefore, the surface charge of a nanoparticle should be designed to be near-neutral for permeability. As the YSK05-MEND is near-neutral at pH7.4, it would be expected that the YSK05-MEND would diffuse in tumor tissue. However, it is widely known that the pH value in tumor tissue is lower than that in healthy tissue and specifically depends on the tumor type and the depth from the nearest blood vessel (Vaupel, 2004). Therefore, the pKa value of ionizable

nanoparticles probably should be adjusted depending on the tumor model in use. Also, as we found that tumor-associated macrophages took up higher amounts of the YSK05-MEND compared to tumor cells in several subcutaneous tumor models (unpublished data), active targeting might be important for achieving not only an improvement in selectivity but also homogeneous drug delivery. However, the affinity for the target receptor also restricts the permeability of nanoparticles under some conditions. It is known that the effective binding of ligand-modified nanoparticles to target receptors in the immediate proximity of tumor blood vessels results in a decline of the number of unbound nanoparticles which can penetrate deep into tumor tissue. This phenomenon is known as the binding site barrier (Fujimori, Covell, Fletcher, & Weinstein, 1990). It has been reported that the distribution of free antibodies against D3 antigens are restricted to the periphery of the antigen-positive patch, whereas the isotype matched IgG readily diffused (Juweid et al., 1992). Also, the permeability of antibody-modified nanoparticles was also limited compared to a nontargeted counterpart (Lee, Fonge, Hoang, Reilly, & Allen, 2010). These results suggest that the amount and distribution of receptors, and a ligand with an adequate affinity should be considered for achieving homogenous and efficient targeting to tumor cells.

5.2.4 Application of YSK05-MEND for Tumor Tissue

siRNA can be used for altering the cell phenotype such as drug resistance. We recently reported on a combination therapy of a cytotoxic drug doxorubicin (DOX) and the YSK05-MEND encapsulating an antipolo-like kinase 1 (PLK1) siRNA (siPLK1) in a renal cell carcinoma (RCC), which is known to be resistant to classical cytotoxic anticancer drugs (Sakurai, Hatakeyama, Akita, & Harashima, 2014). As it is known that the inhibition of PLK1 can induce apoptosis in various tumor cells, PLK1 is a promising target for cancer therapy. Administration of the YSK05-MEND resulted in a PLK1 gene silencing of approximately 60%, but failed to induce a significant antitumor effect in vivo, indicating that RCC is resistant to PLK1 inhibition. At the same time, PLK1 inhibition led to the down regulation of cyclin B1 (CCNB1), which is one of the target proteins for the PLK1 gene. CCNB1 plays a pivotal role in the G2/M phase transition, and it has been reported that cells in the G2/M phase are more sensitive to DOX compared to cells in the G1 phase. Taken together, PLK1 gene silencing should lead to a decline in CCNB1 expression, cell cycle arrest in the G2/M phase and sensitization against DOX. A combination of a liposome encapsulating DOX and a YSK05-MEND encapsulating siPLK1 induced apoptosis and showed a significant antitumor effect in RCC-bearing

mice. This result suggests that the combination therapy of siPLK1 and DOX can be a potentially useful strategy for developing a new treatment for RCC, and the use of the YSK05-MEND is an attractive method for the analysis of cancer biology in vivo.

As angiogenesis is essential for tumor growth and metastasis, targeting angiogenesis (antiangiogenic therapy) is a rational strategy for the treatment of cancer. Based on this, a monoclonal antibody against vascular endothelial growth factor (VEGF) is currently being used in the treatment of various types of cancer. The cyclo (Arg-Gly-Asp-D-Phe-Lys) (cRGD) peptide, a ligand for $\alpha_v\beta_3$ integrin which is highly expressed on the surface of TECs, is used for targeting TECs. We demonstrated that the cRGD-modified YSK05-MEND encapsulating the anti-VEGF receptor 1 (VEGFR1) selectively bound to TECs and induced VEGFR1 gene silencing and an antitumor effect after intravenous injection. We recently succeeded in improving the gene silencing activity of the YSK05-MEND on TECs by approximately 10 times through an examination of the lipid composition and ligand modification. Also, the importance of TECs on the construction of the tumor microenvironment has been recognized through an understanding of genetic and functional abnormality of TECs. Taken together, the cRGD modified YSK05-MEND promises to contribute a further understanding of the role of TECs and antiangiogenic cancer therapy.

6. SS-CLEAVABLE PROTON-ACTIVATED LIPID-LIKE MATERIAL (SSPALM)

6.1 Quantitative Analysis of Intracellular Trafficking between Adenovirus and Lipoplex

For the development of new-generation gene carriers, it is necessary to understand what the weak point of the current nonviral carrier is. In parallel with the evolution of life forms, viruses have evolved and developed an elegant machinery to deliver their genome DNA into the nucleus. If we could understand why and to what extent current nonviral vectors are inferior to the viral ones, such information would be quite valuable in terms of developing the next generation of gene carrier. We utilized various types of imaging equipment to analyze intracellular trafficking. First, we proposed a strategy for quantifying the simultaneous distribution of pDNA in the cytosol, endosomes/lysosomes and the nucleus, using sequential Z-series images captured by CLSM, a technique that is referred to as Confocal Image-assisted 3-Dimensionally Integrated Quantification (CIDIQ)

(Akita, Ito, Khalil, Futaki, & Harashima, 2004). This method, along with quantitative PCR, was used to compare the intracellular trafficking of adenovirus and cationic lipoplex (Lipofectamine Plus: LFN).

Concerning transfection efficacy, LFN requires 3–4 orders of magnitude more gene copies than adenovirus to achieve comparable transgene expression. Thus, we originally assumed that the intracellular processes (endosomal escape or nuclear delivery) of LFN would be quite poor in comparison with adenovirus. However, quantitative analysis ultimately revealed that the nuclear transport efficiency of adenovirus was only twofold greater than that of LFN. Thus, the large difference in transfection efficiency cannot be explained by intracellular trafficking. In other words, a postnuclear delivery process is primarily responsible for the overall difference in transfection efficiency between LFN and adenovirus. In fact, the postnuclear delivery efficiency corresponding to the transfection activity divided by the nucleus-delivered gene copies were 8100-fold higher in adenovirus in comparison with LFN. This conclusion was also supported in other types of polycation or lipoplex (Cohen, van der Aa, Macaraeg, Lee, & Szoka, 2009; Varga et al., 2005).

6.2 Mechanism for the Poor Postnuclear Processes in Lipoplex

The postnuclear delivery process is, in turn rate-limited by transcription and translation. Thus, the contributions of these two processes to the overall differences in the postnuclear delivery processes were quantitatively evaluated by measuring mRNA expression. Transcription efficiency can be calculated as mRNA copies divided by nuclear DNA. Similarly, translation efficiency was calculated as transgene expression divided by mRNA copies. As a result, a three order of magnitude difference in the postnuclear delivery process was found, which can be attributed to the 1 order of magnitude difference in the transcription process and 2 orders of magnitude difference in the translation process (Hama, Akita, Iida, Mizuguchi, & Harashima, 2007). Our recent study indicated that a poor postnuclear delivery process is also a key factor for the heterogeneity of transgene expression in nonviral vectors (Akita, Ito, Kamiya, Kogure, & Harashima, 2007; Akita, Umetsu, Kurihara, & Harashima, 2011). Collectively, these findings clearly indicate that an improvement in post-nuclear events is essential for achieving a transgene expression comparable to that for the viral vector, and resolve heterogeneity.

To use these results as feedback for the development of a nonviral carrier, it is necessary to understand why the postnuclear delivery process is poor in the case of LFN. To gain insights into the poor transcription efficacy in LFN, we visualized the DNAs (adenovirus genome DNA and pDNA) in

the nucleus by means of in situ hybridization to compare their intranuclear disposition. The findings confirmed that the ODN probe can bind to the target sequence in naked DNA, but not in the lipoplex because of steric hindrance. Thus, in situ hybridization can selectively detect DNAs that were released from the gene carrier. As a result, the pDNA signal was less prominent compared with the adenoviral genome, even though the total amount of nuclear DNA was higher in LFN compared with an adenoviral transfection. These results strongly suggest that poor decondensation is responsible for the less efficient nuclear transcription with LFN.

Concerning the mechanism responsible for the prominent differences in translation, we found that the in vitro translation process was drastically inhibited in the presence of LFN, whereas the inhibitory effect of adenovirus was negligible. Thus, the poor translation efficiency for LFN can be explained by electrostatic interactions between the positively charged cationic liposomes in LFN and negatively charged mRNA.

In summary, a novel strategy for controlling the intranuclear decondensation and to minimize electrostatic interactions between mRNA and vectors is needed to further improve the efficiency of nonviral vectors. As to the intranuclear decondensation, the FRET between donor fluorophores labeled in pDNA and its acceptor in the carrier can be useful to visualize this process (Shaheen, Akita, Yamashita, et al., 2011).

6.3 Rational Design of a Particle to Overcome the Drawbacks Associated with Cationic Carriers (MENDssPalm)

Since relatively high concentrations of negatively charged constituents, such as HSPGs are located on the surface of the plasma membrane, many of the previous vectors were designed to carry a high level of positive charges, by the extensive condensation of pDNA with cationic liposomes and polycations under the assumption that cationic charge is a crucial driving force for the first cellular contact with the HSPG, and the subsequent cellular uptake process. However, quantitative comparisons of intracellular trafficking between cationic nonviral vectors (lipoplex and polyplex) revealed that the poor postnuclear delivery processes can be directly attributed to the inefficiency associated with transcription and translation. This, in turn, can be attributed to the poor release of pDNA from the gene carriers, and to the steric hindrance likely due to electrostatic interactions between mRNA and the cationic component of the gene carrier (i.e., cationic liposomes), respectively (Hama et al., 2007; Hama et al., 2006).

Based on these drawbacks, we focused on the development of a pDNA-encapsulating nanoparticle which was designed to be neutral at physiological

(cytoplasmic) pH to avoid mRNA interactions, and to be degradable, resulting in the effective release of pDNA in response to the cytoplasmic environment. As a molecular design to overcome these drawbacks, a neutral lipid envelope-type MEND was developed. A key molecular component is an SS-cleavable Proton-Activated Lipid-like Material (ssPalm) that is comprised of three functional units; (1) tertiary amine groups for positive charging in only an acidic environment in endosomes/lysosomes to permit endosomal escape, (2) disulfide bonding to permit reductive cleavage in response to the cytoplasm, and/or the disulfide reductase on the cell surface, (3) hydrophobic molecules to permit the formation of a lipid envelope structure (Akita, Ishiba, et al., 2013) (Figure 6.12). In initial experiments, myristic acid (C_{14}) was employed (ssPalmM). By the cleavage of the

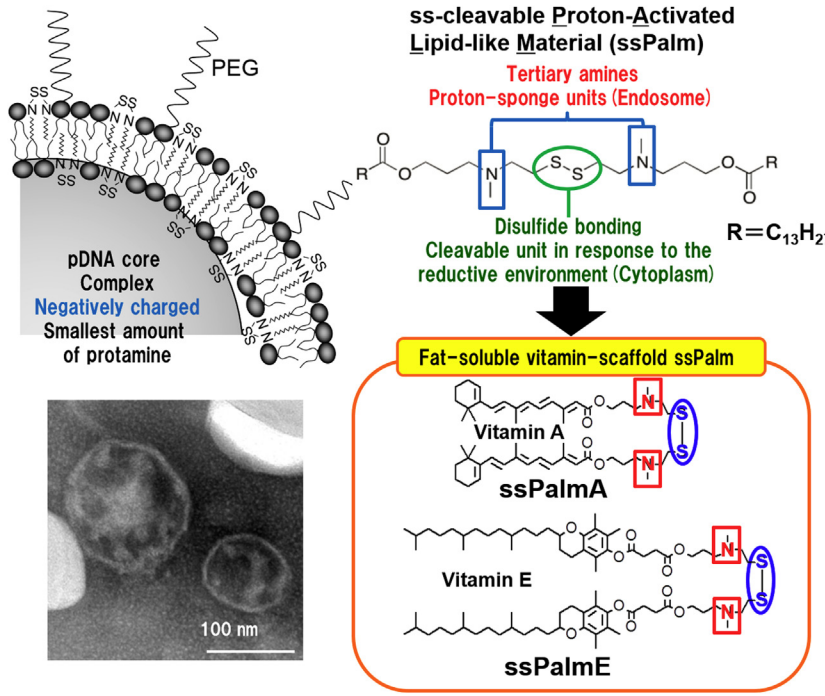

Figure 6.12 ***Nanoparticle prepared with SS-cleavable and Proton-Activated Lipid-like Material (ssPalm) functions as a key element in a lipid nanoparticle in which pDNA is encapsulated.*** The ssPalm mounts dual sensing motifs that can respond to the intracellular environment; a proton-sponge unit (tertiary amines) that functions in response to an acidic environment (endosome/lysosome), and disulfide bonding that can be cleaved in a reducing environment (cytosol). As a second generation of ssPalm, fat-soluble vitamins were used as a hydrophobic scaffold.

disulfide bonding, the ssPalm with two-hydrophobic chains were converted into a product with one hydrophobic chain that functions as a surfactant. Thus, it triggers the destabilization of the envelope structure.

The MEND prepared with the ssPalmM ($MEND_{ssPalmM}$) showed a higher gene transfection activity in the presence of serum, in comparison with MENDs that were prepared with DODAP, a pH-sensitive lipid with a tertiary amine ($MEND_{DODAP}$), or a noncleavable Palm-derivative, in which disulfide bonding was replaced with carbon bonding ($MEND_{ccPalmM}$) (Akita, Ishiba, et al., 2013). Of note, the activity of $MEND_{ssPalmM}$ was comparable to a positively charged MEND prepared with DOTAP, a cationic lipid containing quaternary amines ($MEND_{DOTAP}$) with less cellular uptake. In other words, the poor cellular uptake was complemented by the well-refined intracellular processes. In fact, the inhibitory effect of the $MEND_{ssPalmM}$ on in vitro translation was much less than that for the cationic $MEND_{DOTAP}$. Moreover, the amount of protein after the in vitro transfection using the $MEND_{ssPalmM}$ remained constant, even when the dose was increased by 10-fold than general transfection condition, whereas the corresponding values in $MEND_{DOTAP}$ or $MEND_{ccPalmM}$ were drastically decreased in a dose-dependent manner. Thus, biodegradable characteristics, along with noncationic features are both important factors for the extremely low cytotoxicity.

Transfection activity was diminished by blocking endosomal acidification by treatment with NH_4Cl or Bafilomycin A. Thus, pH-activated positive charging is prerequisite for the destabilization of endosomes in the $MEND_{ssPalmM}$, and this occurs by triggering the formation of electrostatic interactions between MEND particles and the endosomal membrane.

To investigate the destabilization and resulting decapsulation of pDNA in the cell, a double-labeled MENDs was prepared, in which the pDNA was labeled with rhodamine and the lipid envelope was labeled with NBD. In the case of the $MEND_{ccPalmM}$, pDNA signals were predominantly colocalized with the lipid envelope signals. In contrast, a significantly larger portion of the pDNA signals were detected free from the signal derived from the envelope in the $MEND_{ssPalmM}$. These data suggest that the decapsulation efficiency was actually improved after the particles had been taken up by the cells, as was intended in the design.

As a second generation of ssPalm, a fat-soluble vitamin such as vitamin A (retinoic acid) or vitamin E (α-tocopherol succinate) was used as a hydrophobic scaffold (ssPalmA and ssPalmE, respectively). The gene expression

of the MEND prepared with ssPalmA ($MEND_{ssPalmA}$) was 15-fold higher than the gene expression of $MEND_{ccPalmM}$ at a maximum, while the cellular uptake of both particles was comparable (Tanaka et al., 2014). Microscopic observations with nucleus-stained cells revealed that the pDNA delivered by the $MEND_{ssPalmA}$ reached the boundary of the nucleus in the form of a lipid-encapsulated particle, and the lipid membrane then became gradually decapsulated, while the pDNA delivered by the $MEND_{ssPalmM}$ was rapidly released from the LNPs and dispersed in the cytoplasm. Moreover, the gene expression and accumulation at the nuclear periphery was drastically impaired by the excess amount of retinoic acid. Thus, it is plausible that $MEND_{ssPalmA}$ was recognized by the nuclear transport machinery of vitamin A (such as the cellular retinoic acid-binding protein II) in the host cells (Budhu & Noy, 2002).

6.4 In vivo Applications of ssPalm for Hepatic Gene Delivery

Since the hepatic blood vessels are organized by fenestrated endothelial cells (Jacobs et al., 2010; Snoeys et al., 2007; Wisse et al., 2008), the liver represents an ideal target organ for gene delivery by means of artificial nanoparticles, as well as naked plasmid DNA (pDNA) (Bonamassa, Hai, & Liu, 2011; Herweijer & Wolff, 2007) or viruses (i.e., adenovirus (Bonamassa et al., 2011) and adeno-associated viruses (van der Laan, Wang, Tilanus, Janssen, & Pan, 2011)).

Cationic liposomes and polycations are frequently used as fundamental materials in artificial gene carriers, since they can efficiently form condensed nanoparticles via electrostatic interactions, thus avoiding enzymatic degradation in the serum. However, positively charged particles that are formed by excess condensation immediately form large aggregates with erythrocytes, which then rapidly accumulate in the lung (McLean et al., 1997; Nomoto et al., 2011; Ogris, Brunner, Schuller, Kircheis, & Wagner, 1999) when they are administered to the systemic circulation in vivo. We also developed a nanoparticle for in vivo hepatic gene delivery, whose critical structural elements are a DNA/protamine condensed core particle, successively coated with lipid bilayers composed of a cationic lipid (*N*-[1-(2,3-dioleyloxy) propyl]-*N,N,N*-trimethylammonium; DOTMA) (Akita, Masuda, et al., 2011), that contains a quaternary amine moiety in its structure. While the hepatic transfection activity of a marker gene (luciferase: GL3) normalized to the total amount of protein (RLU/mg protein) was increased by the modification of the devices; with the GALA peptide as an inducer of endosomal

escape and maltotriose for the enhancement of nuclear delivery (GALA/Malto-MEND), a comparable level of gene expression was also achieved in the lung and spleen. Collectively, the poor tissue selectivity in the liver is one of the major drawbacks to overcome in developing a conventional artificial gene carrier. As another type of liver-targeting MEND, we developed the R8/GALA-MEND as a liver-targeting transgene expression as described above (Khalil et al., 2011).

Another drawback of the traditional gene vector is the short duration of the gene expression. After the i.v. or PO administration of a cationic lipoplex or polyplex, in vivo gene expression generally peaked at approximately 6 h, and then rapidly decreased within 24 h after administration (Fumoto et al., 2004; Kawakami, Ito, Charoensit, Yamashita, & Hashida, 2006). While the mechanism responsible for this remains elusive, the inflammatory response might be related to the silencing of in vivo gene expression. Hyde et al. reported that the complete deletion of unmethylated CpG dinucleotide (CpG) motifs from the plasmid DNA (pDNA) conferred a sustained gene expression with minimal inflammatory response in the lung after in vivo aerosol delivery as a cationic lipoplex (Hyde et al., 2008). Therefore, the use of pDNA that is devoid of CpG motifs is generally considered to be a promising strategy for prolonging the duration of gene expression (Hattori et al., 2010; Hodges, Taylor, Joseph, Bourgeois, & Scheule, 2004; Mitsui et al., 2009).

For in vivo applications of the ssPalm, a high stability of the particle in the serum is an important feature. In fact, the pDNA or a compacted particle prepared using protamine (charge ratio = 1.2) were rapidly degraded within 1 h in 90% mouse serum. However, once they are encapsulated in the $MEND_{ssPalmM}$, the pDNA remains intact for at least 24 h (Akita, Ishiba, et al., 2013). Furthermore, intravital real-time imaging revealed that the $MEND_{ssPalmM}$ did not form aggregates in liver capillaries, and was rapidly taken up by hepatocytes within 10 min, while conventional cationic carriers (R8-MEND) were trapped within the hepatic endothelium in the form of large aggregates. Furthermore, a sustained and liver-specific gene expression (up to 2 weeks) was achieved via the i.v. administration of the $MEND_{ssPalmM}$ encapsulating pDNA free from unmethylated CpG motifs, while gene expression of that encapsulating CpG-rich pDNA diminished within 1 day. Hepatic gene expression also decreased rapidly within 1 day when a conventional cationic liposomal carrier (R8/GALA-MEND or in vivo JetPEI-Gal) was injected, even when it carried CpG-free pDNA (Akita, Ishiba, et al., 2013).

A previous study demonstrated that the persistence of transgene expression is inversely correlated with inflammatory responses in pulmonary tissue (Hyde et al., 2008). In addition, an in vitro transfection study demonstrated that transgene expression efficiencies that are driven by various types of promoters are reduced by treatment with TNFα and/or IFNγ. In addition, a prolonged transgene expression was achieved by the administration of a neutralizing anti-INFγ monoclonal antibody (Qin et al., 1997). Furthermore, the sequential i.v. administration of cationic liposomes and pDNA-enhanced transgene expression in the lungs with a reduced production of cytokines (TNFα, IL12 and IFNγ) compared with simultaneous injection in the form of a lipoplex (Tan, Liu, Li, Li, & Huang, 2001). Therefore, we assumed that the cytokine production triggered by the administration of these particles might be a fundamental event for the silencing of transgene expression. Consistent with these hypotheses, inflammatory cytokines (TNFα, IL-12, and INFγ) were produced at high levels in MENDs exhibiting a short duration of gene expression ($MEND_{ssPalmM}$ encapsulating CpG-rich pDNA, or R8/GALA-MEND encapsulating CpG-free pDNA). Finally, it should be emphasized that the $MEND_{ssPalmM}$ encapsulating CpG-free pDNA is capable of avoiding all kinds of inflammatory responses that were tested. Therefore, the persistent gene expression associated with this particle might be closely related to its ability to avoid immune responses by virtue of its stealth activity. The collective data indicate that the neutralized $MEND_{ssPalmM}$ is a promising hepatic pDNA carrier for sustained and liver-specific gene expression when it carries CpG-free pDNA.

7. PERSPECTIVES

We have been developing MENDs as a concept for intelligent drug delivery system inspired by viral vectors. Controlled intracellular trafficking as well as biodistribution have been improved remarkably compared to the level of studies reported in the twentieth century. Further progress will be made continuously for each system and our objective is to reach the level of a nanomachine, our ultimate goal. At the same time, it is important for some of these materials to proceed to the stage of clinical trial for a novel nanomedicine, however, a "Death Valley" between university laboratories and the pharmaceutical industry exists. A key factor will be how to make nanocarriers under the level of GLP/GMP to proceed to the stage of nonclinical testing. We believe that we can build a rainbow bridge over this valley by encouraging collaboration among universities, pharmaceutical companies, and government institutions in the near future.

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CHAPTER SEVEN

Lipid-Coated Calcium Phosphate Nanoparticles for Nonviral Gene Therapy

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Contents

Abstract

Though calcium phosphate (CaP) nanoparticles have been utilized as transfection agents in laboratory settings for nearly half a century, their power as a platform for therapeutically focused approaches has only recently been realized. With regard to modern advances in lipid- and polymer-mediated transfection, we present liposome CaP, a novel combination of a stable yet acid-labile CaP core with a surface functional, asymmetric lipid bilayer. Herein, the structural and functional dynamics of such nanoparticles are described in detail, including alteration of hepatocellular tropism through PEGylation and a multifunctional approach to endosomal escape, along with primary therapeutic advances, which these nanoparticles contribute to the field overall. Through these and other multifunctional nanotechnologies for gene therapy, great

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promise is shown in their ability to tackle truly complex and mechanistically demanding diseases.

1. RECENT PROGRESS OF NONVIRAL GENE THERAPY AND *In vitro/In vivo* DELIVERY SYSTEMS

Of the numerous efforts within the field of nanotechnology in drug delivery, systemically mediated gene therapy remains the most mechanistically complex, involving over a dozen parameters at minimum, which must be optimized in order to achieve meaningful efficacy. First, a particle system must be developed that can both effectively and stably encapsulate the drug of interest while avoiding aggregation in solution (Hu, Haynes, Wang, Liu, & Huang, 2013; Huang & Liu, 2011; Klibanov, Maruyama, Torchilin, & Huang, 1990). Upon injection (most commonly through the intravenous or subcutaneous routes), the formulation must remain either dispersed or mechanistically localized in the high salt and protein conditions of the blood, avoid off target distribution as well as renal and hepatic clearance, and remain relatively unobserved by the immune system (Choi et al., 2007; Huang & Liu, 2011; Klibanov et al., 1990; Liu, Mori, & Huang, 1992; Wong et al., 2012). Nuclease-mediated degradation must be limited, accumulation at the site of interest must be maximized, and effective means for cellular internalization should be engendered into the delivery system due to the high charge and molecular weight inherent to nucleic acid therapies (Banerjee, Tyagi, Li, & Huang, 2004; Haynes & Huang, 2013; Panyam & Labhasetwar, 2003; Wong et al., 2012; Wooddell et al., 2013). Upon endocytosis, formulations designed in part to entrap their cargo must paradoxically release it and allow for efficient escape into the cytosol (Hafez, Maurer, & Cullis, 2001; Lee, Yang, Kao, Pierce, & Murthy, 2009; Sonawane, Szoka, & Verkman, 2003). Further, with regard to transgene expression, trafficking to and import into the nucleus, release of the free vector, and stimulation of gene expression may need to be addressed as well (Hu et al., 2013; Khalil, Hayashi, Mizuno, & Harashima, 2011; Mudhakir & Harashima, 2009). Such a tremendous stepwise progression leaves much to be done, but much to be improved upon as well. In recent years, the field has exhibited tremendous flexibility in multifunctional formulation development and performance optimization in order to tackle these seemingly disparate requirements; a number of notable systems will be highlighted below.

Polymer-based approaches (Figure 7.1(A)) often attempt to advance upon polyethyleneimine (PEI) as a fundamental carrier system for gene delivery applications (Fortune, Novobrantseva, & Klibanov, 2011; Liang et al., 2008); copolymer-based approaches with antifouling materials such

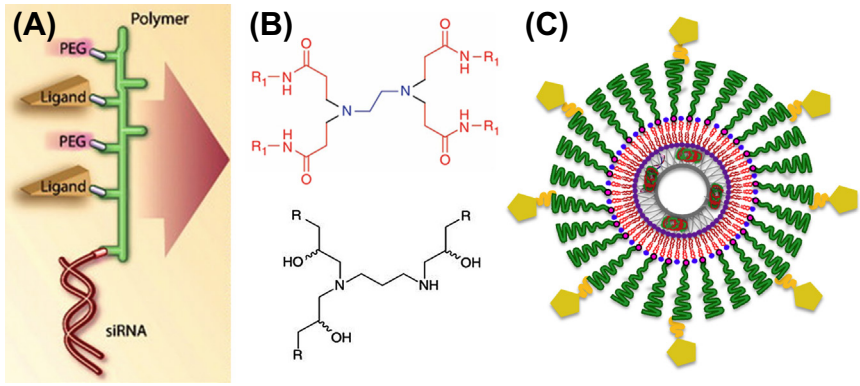

Figure 7.1 ***A sampling of nonviral vectors for gene delivery.*** (A) Dynamic PolyConjugate, employing a grafted design of antifouling molecules, targeting ligands, and therapeutic RNA built off of a poly(butyl/amino vinyl ether) (PBAVE) polymer backbone. (B) Model lipidoids, synthesized via either Michael addition or epoxide chemistry. (C) LCP, a calcium phosphate core supporting an asymmetric, cationic, and PEGylated lipid bilayer, decorated at the surface with targeting ligands. PEG, polyethylene glycol. (See the color plate.) *Adapted with permission from Akinc et al. (2008), Hu et al. (2013), Rozema et al. (2007), Love et al. (2010).*

as poly(ethylene glycol) (PEG) have been widely explored (Kleemann et al., 2005; Knop, Hoogenboom, Fischer, & Schubert, 2010; Liang et al., 2008) and acid-degradable (Lee et al., 2009; Liu, Jiang, Zhou, Zhang, & Saltzman, 2011) as well as bioreducible (Kim, Ou, Lee, & Kim, 2009; Lin et al., 2006) polymer delivery systems have been developed for such purposes, exploiting cationic interactions with the cell membrane as well as changes in the intracellular environment to mediate cargo release. One alternative system, Dynamic PolyConjugate, exhibits significant degrees of deliberate multifunctionality: grafted off of a poly(butyl/amino vinyl ether) backbone are therapeutics (siRNA), antifouling agents (PEG), and targeting moieties (n-acetylgalactosamine) optimized for hepatic gene silencing to dramatic degrees (Rozema et al., 2007). Building off of the successes of stable nucleic acid lipid particles (Zimmermann et al., 2006), modern lipid-based methods (Figure 7.1(B)) have taken approaches from combinatorial chemistry in development, in that extensive libraries of synthetic lipid-like materials, termed "lipidoids," have been developed and explored for optimal parameters by which to maximize gene silencing (Akinc et al., 2008; Love et al., 2010). Selective ionization of lipid surfaces has been explored as well in order to control for intracellular release and minimize systemic recognition prior to target distribution (Semple et al., 2010). Further efforts seek instead to address recently recognized problems created by some of the field's most ubiquitous solutions. The PEG dilemma has been widely discussed

(Huang & Liu, 2011; Birgit Romberg, Hennink, & Storm, 2008), in that the means by which to promote circulation and maximize desired distribution (commonly, PEGylation) can conversely limit cell uptake and cargo release at the site of interest. In this vein, sheddable PEG systems supported by either pH-sensitive (Li, Huang, MacKay, Grube, & Szoka, 2005), reduction-sensitive (McNeeley, Karathanasis, Annapragada, & Bellamkonda, 2009), or enzyme-sensitive (Wong et al., 2011) bonds have been developed for the purposes of selective delivery. The phenomenon of accelerated blood clearance of liposomes upon repeated injection due to immune production of anti-PEG IgM has also been described (Ishida & Kiwada, 2008; Shimizu, Ishida, & Kiwada, 2013), with corresponding efforts to develop alternative antifouling materials (e.g., poly(vinyl pyrrolidone) (PVP), poly(hydroxyethyl-l-asparigine) (PHEA)), which markedly reduce such effects (Ishihara et al., 2010; Knop et al., 2010; Romberg et al., 2007).

Nevertheless, synthesis of complementary approaches into a multifunctional platform, which addresses each parameter inherent to the gene delivery process remains the ideal to which all nanosystems currently strive (Hu et al., 2013). Herein, we will emphasize our group's specific contributions to the art, but after giving essential credence to the formulation subdiscipline upon which our nanoparticle library is based. CaP-based delivery methods are still commonly utilized in in vitro settings for simple and straightforward transfection through in situ precipitation under the control of a variety of parameters such as pH, mixing, and reagent concentrations (Epple et al., 2010). They provide a direct means for encapsulation of nucleic acids in a solid precipitate state (Tabaković, Kester, & Adair, 2012) and hold benefits for intracellular release due to decreased intracellular calcium and phosphate concentrations (Epple et al., 2010) as well as sensitivity to reduced endosomal pH (Haynes & Huang, 2013; Huang & Liu, 2011; Li, Chen, Tseng, Mozumdar, & Huang, 2010). However, toxicity concerns are still debated by many (Luo & Saltzman, 2000), and rapid crystal growth is often difficult to control, limiting its advancement into more complex settings (e.g., *in vivo*) (Epple et al., 2010; Luo & Saltzman, 2000). Methods by which to limit such growth have been developed, most notably by Kataoka and colleagues (Kakizawa, Furukawa, & Kataoka, 2004; Kakizawa & Kataoka, 2002; Pittella et al., 2011) through the use of amphiphilic block copolymers to abrogate crystal propagation, yielding narrowly disperse and therapeutically efficacious formulations. Building upon such approaches, we present our novel lipid bilayer-supported CaP nanoparticles, referred to as liposome CaP (LCP, Figure 7.1(C)), which employ multifunctional physicochemical characteristics

to achieve dramatic efficacy in areas such as transgene expression, cancer therapy, and treatment of liver disease. CaP is well known to be an acid-labile delivery system (Epple et al., 2010; Li et al., 2010) whose nanoprecipitation process performed within the microemulsion setting has proven highly efficient (50% or greater) at encapsulating a variety of RNA and DNA vectors (Hu et al., 2013; Li et al., 2010; Xu, Wang, Zhang, & Huang, 2014), proteins, and peptides (Xu et al., 2013), as well as phosphorylated prodrugs (Zhang, Schwerbrock, Rogers, Kim, & Huang, 2013). Such contents, encapsulated on the nanoscale, can thus be rapidly and efficiently released in the acidic endo/lysosomal environment of the cell (Haynes & Huang, 2013; Huang & Liu, 2011). Further, the development of a stable monolayer of dioleoyl glycero-phosphate (DOPA) on the surface of the CaP nanoparticles allows for the unique construction of an asymmetric lipid bilayer, in that the outer leaflet lipids incorporated into the rehydration process neither must involve DOPA nor exchange with the DOPA monolayer upon rehydration (Li, Yang, & Huang, 2012). Such flexibility supports the use of common lipid-based methods for cellular uptake and transfection (cationic lipids such as 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)) as well as minimized aggregation potential and increased circulation longevity distearoyl phosphatidylethanolamine (DSPE-PEG). Cationic lipids on the outer leaflet further potentiate endosomal disruption (described in a later section), while PEGylation provides a straightforward means for targeting through simple distal chain end conjugation of various ligands (galactose, anisamide) (Banerjee et al., 2004; Hu et al., 2013). A proposed scheme for gene transfer through delivery by LCP is shown in Figure 7.2.

In this chapter, the formulation process will be described in detail, emphasizing important structural characteristics when necessary. From there, brief expansion will be given to the various means by which LCP supports a multifunctional approach to drug and gene delivery. Specific mechanistic utilities that are unique to LCP and similarly structured liposome-coated nanoprecipitates will be highlighted; specifically, extensive degrees of liposome PEGylation, a multifaceted approach to endosomal escape, and a novel means by which to support enhanced nuclear translocation of plasmid DNA and subsequent transgene expression. While diverse therapeutic applications will be explored in other chapters, this chapter will focus primarily on hepatic transgene expression as well as siRNA-based efforts in cancer therapy and the treatment of nonalcoholic fatty liver disease (NAFLD).

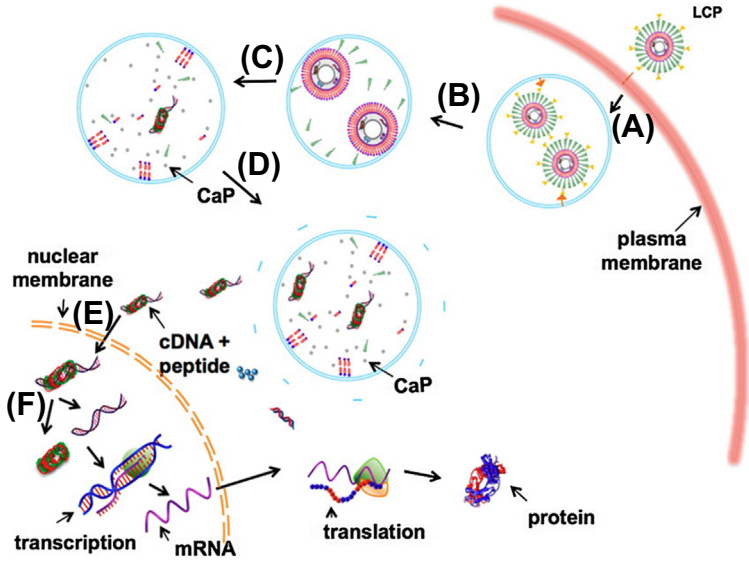

Figure 7.2 ***Proposed mechanism for intracellular delivery of DNA by LCP.*** (A) ASGPR-mediated endocytosis, elicited by binding to galactose ligands on LCP, supports internalization into hepatocytes. (B) PEGylation shedding is driven by a decrease in endosomal pH. (C) Dissolution of the calcium phosphate core and breakdown of the lipid membrane promotes (D) disruption of the endosome in a cationic lipid-mediated manner. (E) Nuclear import of DNA is thought to be mediated by cysteine-flanked octa-arginine peptides. (F) DNA is released in the nucleus for active transcription. CaP, calcium phosphate; ASGPR, asialoglycoprotein receptor. *Reprinted with permission from Hu et al. (2013).*

2. LCP NANOPARTICLES AS A MULTIFUNCTIONAL PLATFORM FOR GENE DELIVERY

2.1 LCP Nanoparticles: Preparation and Structure

Liposome calcium phosphate (LCP) is uniquely formulated through a combination reverse-microemulsion process (Figure 7.3(A)). Through the use of a strong surfactant (Igepal-co-520), two separate emulsions in cyclohexane of high calcium chloride and dibasic sodium phosphate are prepared under stirring. Such methods have been used previously to establish solid CaP nanoparticles (Altınoğlu et al., 2008; Roy, Mitra, Maitra, & Mozumdar, 2003); however, the addition of DOPA to the phosphate-based emulsion and subsequent mixing of the two emulsions allows for coating of the CaP nanoparticles with a stable lipid monolayer (Li et al., 2012). Disruption of the microemulsion and centrifugal purification of such LCP "cores" with

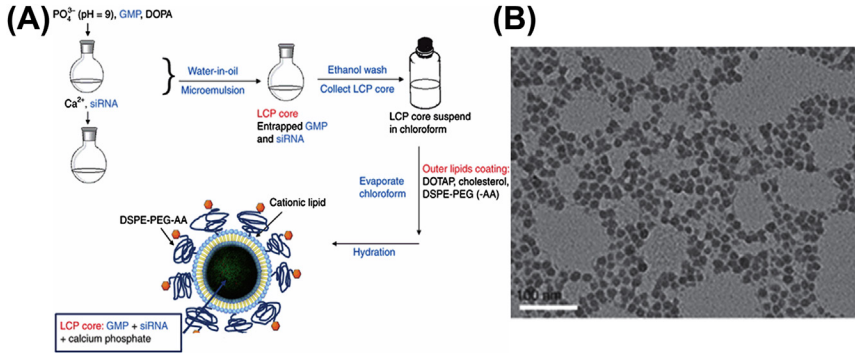

Figure 7.3 (A) Formulation and structural characteristics of LCP, shown herein encapsulating both a small-molecule prodrug (GMP) and interfering RNA. Calcium and phosphate reverse microemulsions are prepared separately and then mixed, followed by centrifugal precipitation and washing in absolute ethanol. Functional "LCP cores" (calcium phosphate nanoparticles coated in a lipid monolayer of DOPA) are well soluble in chloroform, whereas poorly coated nanoparticles can be pelleted and removed. Such LCP cores are readily amenable to thin-film rehydration processes as well, yielding the above LCP particles (shown here targeted to sigma receptors via anisamide ligands, AA). (B) TEM image of LCP nanoparticle cores. GMP, gemcitabine monophosphate. *Adapted with permission from Zhang, Schwerbrock, et al. (2013).*

absolute ethanol provides means for high recovery, after which the cores can be easily resuspended in chloroform. A final centrifugation removes large aggregates from the purified product, yielding an optimized batch of CaP particles, which are efficiently and completely coated with a lipid monolayer of DOPA. Such cores are stable at −20 °C for upward of 1 year (Haynes & Huang, 2013; Hu et al., 2013; Huang & Liu, 2011; Li et al., 2010).

Further, LCP cores are readily amenable to the prototypical thin-film rehydration process common to a variety of liposome formulations (Li et al., 2010; Tan, Whitmore, Li, Frederik, & Huang, 2002). LCP formulations typically employ a subset of cationic and helper lipids (DOTAP, dioleoyl phosphatidylcholine (DOPC)), stabilized by cholesterol (Chol), and dispersed effectively by means of PEGylation (DSPE-PEG_{2000}), which are mixed with the core solution, dried under nitrogen gas and desiccated, and subsequently rehydrated under decreasing concentrations of ethanol, producing (via light vortexing and sonication) what are commonly referred to as "final" LCP nanoparticles—a CaP core, encapsulated by a lipid bilayer, coated on its outer leaflet by both cationic and PEGylated lipids (Figure 7.3(B)). Such nanoparticles are stable for 2–3 days at 4 °C, while further amenable to lyophilization for prolonged storage. In the following sections, we will highlight mechanisms of importance to

LCP delivery *in vitro* and *in vivo*, and describe the potent means by which LCP enhances the multifunctional approach to nonviral gene therapy.

2.2 PEGylation of LCP for Enhanced Circulation Longevity and Cellular Tropism *in vivo*

The use of PEG in modification of therapeutics and nanoparticles ("PEGylation") has elicited dramatic results in terms of global pharmacokinetics and biodistribution. In many cases, small-molecule and protein drugs can be rapidly cleared from the circulation via renal filtration as well as transversal of the capillary endothelium; provision of a hydrodynamic (HD) diameter of greater than 5–10 nm supports vascular retention of such materials and can prolong one's overall exposure (Ishida, Maruyama, Sasaki, & Iwatsuru, 1999). Since its inception in lipid modification, PEGylation has made a similar impact on liposome circulation time *in vivo*; its implications within the drug delivery process in both maximizing the enhanced permeation and retention effect and minimizing mononuclear phagocyte system recognition are described heavily in previous chapters (Huang & Liu, 2011). To again define the function of PEG more clearly, as it possesses few molecular handles with which to bind to other structures (i.e., lacking ionic components, hydrogen bond donors), each PEG molecule is hypothesized to produce what is known as a "conformational cloud" on the nanoparticle surface. In the local free volume around each PEG chain, rapid chain mobility supports the exploration of that free volume such that adsorbent materials, such as albumin and other serum proteins, are sterically excluded from the liposome surface (Haynes & Huang, 2013; Huang & Liu, 2011; Torchilin et al., 1994). At sufficiently high surface densities (wherein the distance between any two PEG chains is less than the Flory radius of the PEG chains (Huang & Liu, 2011)), the free volumes of these PEG chains begin to overlap; in other words, the PEG chains begin to interact and drive conformational exploration upward, achieving a transition from a "mushroom"-like conformation to a "brush"-like conformation. Such a dense barrier to opsonization (adsorption of proteins such as antibodies, complement, and fibronectin) thus limits the potential for immune recognition of PEGylated liposomes, conferring in part the increases in circulation time observed in such nanoparticle systems (Haynes & Huang, 2013; Huang & Liu, 2011). Various polymeric materials have been developed (e.g., alcohols (Takeuchi, Kojima, Yamamoto, & Kawashima, 2000), saccharides (Mobed & Chang, 1998), amino acids (Metselaar et al., 2003)), and issues regarding PEGylation as a stealth system have been reviewed extensively

(Knop et al., 2010); however, the material's widespread utility provides a relevant comparative basis for formulation development.

One of the physical parameters most unique to LCP relates to one's ability to explore liposome PEGylation under the concept of what is known as the "supported bilayer," as described in Chapter 4 (Li & Huang, 2009). While free liposomes are unable to support greater than roughly 6 mol% PEGylated lipids in formulation, dissociating into lower-order micellar structures (Huang & Liu, 2011; Silvander, Johnsson, & Edwards, 1998; Uster et al., 1996), liposome polycation/DNA (LPD) was shown able to support up to 10–11 mol% (Li & Huang, 2009). In this fashion, as observed through a fluorescence-based method in purification of LCP from free lipid materials by means of sucrose gradient centrifugation, LCP supports a maximum ratio of 20 mol% PEG-lipids on the nanoparticle surface; such increases in PEGylation can distinctly influence and elongate the elimination phase of the LCP pharmacokinetic profile as well (Liu, Hu, & Huang, 2014). Further, fully optimized LCP nanoparticles exhibit hepatic tropism on both a whole organ and a cell-specific level. Upon distribution into the primary capillaries of the liver, nanoparticles are impeded by the hepatic sinusoidal epithelium, which directly separates the plate-like organization of hepatocytes, along with local lymphatics ("space of Disse"), from the peripheral vasculature. Within these epithelia are pores ranging from 100 to 175 nm in diameter, organized in a fashion representative of "sieve plates," which allow for passive exposure of materials between the vasculature and the hepatocytes (Crispe, 2003). Under such conditions, the sufficiently small and highly PEGylated LCP nanoparticles present as an ideal platform for local delivery to hepatocytes (Haynes & Huang, 2013). The high degree of PEGylation, which LCP supports also dramatically influences the local biodistribution of such nanoparticles, promoting Kupffer cell uptake at lower PEG densities (less than 10 mol%) while transferring uptake predominance to primary hepatocytes at higher PEG densities (15–20 mol%) (Liu et al., 2014). Thus, the degree of liposome PEGylation may influence not only the nanoparticle circulation longevity, but also its local biodistribution—an important consideration in the cell-specific delivery of complex therapeutics such as siRNA and proteins.

2.3 Mechanisms of Active Cellular Internalization and Controlled Endosomal Release for LCP

Nevertheless, it has been observed through radio-labeling experiments, the global distribution of LCP predominantly favors the spleen under passive conditions (Hu et al., 2013). Thus, in an effort to improve hepatic tropism

and further motivate selective uptake into hepatocytes beyond the passive interactions employed by cationic surface lipids, active targeting strategies were explored. Our lab has explored targeted delivery in cancer therapy through the sigma receptor-specific anisamide ligand (Banerjee et al., 2004); however, the predominant means of active hepatocyte targeting involves carbohydrate-based ligands (e.g., galactose, *n*-acetylgalactosamine). In addition, various lipidic (such as Chol or vitamin E) and lipoprotein-based ligands have also been utilized (Cho et al., 2001; Choi, Liu, Park, & Kim, 1998; Rensen et al., 1997). LCP, optimized for hepatic gene transfer, borrows from such developments through a straightforward amide coupling reaction to the distal chain end of carboxyl-PEG through an aminophenyl galactopyranoside conjugate (Hu et al., 2013). Such nanoparticles, sparsely (1:5 PEG molar ratio) coated with such targeted PEG-lipids (DSPE-PEG-GAL), were shown to greatly enhance overall distribution to the liver and the rate at which LCP distributes to the liver, while motivating receptor-mediated endocytosis through interaction with the asialoglycoprotein receptor (ASGPR) of the hepatocytes (Hu et al., 2013). It should be noted as well that, while it is known that Kupffer cells share affinity toward galactose-bearing nanoparticles through their galactose particle receptors, such affinity is predominantly concerning nanoparticles of dramatically larger sizes, further adding to LCP's hepatocellular selectivity (Popielarski, Hu-Lieskovan, French, Triche, & Davis, 2005; Rahman, Cerny, Patel, Lau, & Wright, 1982).

Effective means for encapsulation and release of drug cargo, as well as improvements in pharmacokinetics, biodistribution, and cellular selectivity, each contributes toward and largely defines the overall efficacy in any standard small molecule delivery platform; however, for therapeutics such as RNA/DNA vectors and many proteins/peptides, which are dramatically limited in their ability to cross cellular membranes, endosomal entrapment of the therapeutic cargoes must be considered as a strong potential limitation on efficacy (Haynes & Huang, 2013). Nanoparticle systems frequently employ materials for high buffering capacity (Kleemann et al., 2005; Liang et al., 2008), membrane destabilization (Khalil et al., 2011; Murthy, Robichaud, Tirrell, Stayton, & Hoffman, 1999), and biodegradation (Kim et al., 2009; Panyam & Labhasetwar, 2003) to mediate such processes. LCP accomplishes this task in a combination fashion: the acid-labile CaP core as well as the cationic and PEGylated outer leaflet lipids may contribute to the disruption of the endosome and release of its contents. Considering

the CaP core, endosomal dissolution due to rapid decreases in pH mediates a notable increase in ion concentrations within both the endosome and, after rupture, the cell as a whole. Such increases likely contribute to endosomal disruption through what is commonly referred to as the "proton sponge" effect. Through materials which can effectively buffer endosomal pH (around 5–7), proton influx into the endosome, coupled with transport of counterions such as chloride, can be driven by such materials to create a strong osmotic gradient toward the endosomal interior. Thereafter, influx of water into the endosome serves to place stress on and rupture the membrane, which cannot compensate effectively for the increased interior fluid pressure. Such a scenario has been widely proposed and experimentally tested (Sonawane et al., 2003; Xu & Szoka, 1996), though other competing factors may influence the degree to which molecular cargo escapes the endosome (Yue et al., 2011). Use of a common fluorescent probe for calcium concentrations, fura-2 acetoxymethyl ester, supports such conclusions upon cellular exposure to LCP, and provides effective means by which to consider nanoparticle degradation and cargo release on the intracellular level (Li et al., 2010). The cationic lipids, however, provide a complementary charge to the predominantly anionic endosomal membrane, allowing for the production of an "ion-pair complex" upon endocytosis and nanoparticle degradation. Such lipid interactions interfere with and drive away from the bilayer structure of the endosome, and this switch toward more preferred lipid conformations can support membrane rupture and the release of cargo as well (Hafez et al., 2001). PEGylated lipids may also support endosomal breakdown in a surfactant-like manner through the same PEG shedding phenomenon employed in nanoparticle degradation (Guo & Huang, 2011). To what degree these parameters are each involved in LCP has yet to be explored; however, their combined influence has proven therapeutically effective over a wide range of systems both in vitro and *in vivo* (Hu et al., 2013; Li et al., 2010; Li et al., 2012; Liu et al., 2014; Xu et al., 2013; Xu et al., 2014; Zhang, Schwerbrock, Rogers, Kim, & Huang, 2013).

2.4 Nuclear Import and Release of DNA for Enhanced Plasmid Transgene Expression

Due to dramatic tropism toward the liver (supported by active targeting), and most specifically to the hepatocytes of the liver (at high PEGylation densities), hepatic gene therapy was explored as a potential means by which LCP could establish meaningful advancements. While gene therapy requires

a functional approach at numerous steps along both extracellular and intracellular delivery pathways in order to exact strong efficacy, in the manner described above, LCP presented as a relevant platform by which to accomplish a variety of these considerations. Nevertheless, nuclear import comprises one step along the delivery process, which remained to be considered in the LCP system. To borrow conceptually from more viral-based delivery means, nuclear import is typically developed as follows. Upon endosomal escape, many viruses traffic along the cell's microtubular projections, often in a dynein-dependent manner, toward the nucleus, and localize near a structure known as the nuclear pore complex (NPC). The NPC represents an organization of ~30 structural proteins termed nucleoporins, which typically limit diffusion-based transport to a particle size of roughly 10 nm in diameter. However, materials of much larger size may penetrate the NPC through an active process mediated in part by carrier molecules known as importins (which, along with exportins, form the subset of nuclear delivery proteins known as karyopherins). Such importins (canonically, importin-α) recognize specific sequences on carrier proteins known as nuclear localization signals (NLS) and, supported by co-delivery with importin-β, can traverse the NPC supporting materials up to 40 nm in diameter (Le Sage & Mouland, 2013; Panté & Kann, 2002). Upon successful translocation, release of the NLS from such importins is mediated in an active process by enzymes known as Ran and CAS, which thereafter exit the nucleus to again exchange for GTP to complete the process (Le Sage & Mouland, 2013). It should be noted as well that a variety of viruses have developed means by which to circumvent such RAN-dependent nuclear import as well, involving complex interactions (e.g., docking, displacement) with specific nucleoporins and nuclear histones (Le Sage & Mouland, 2013; Trotman, Mosberger, Fornerod, Stidwill, & Greber, 2001). By comparison, in the development of synthetic systems for gene transfer, consideration has been given toward cationic oligopeptide sequences employing a variety of lysine, arginine, and histidine residues (Kim et al., 2009; Manickam & Oupický, 2006; McKenzie, Smiley, Kwok, & Rice, 2000; Won, Kim, Lee, & Kim, 2010), and potential for enhanced nuclear localization by such means has been suggested; however, significant limitations with regard to endosomal escape (likely due to poor buffering capacities) have been attributed to their low efficiencies in transfection (Melikov & Chernomordik, 2005).

Therein, the coupling of the potential improvements in nuclear localization supported by specifically structured and highly cationic peptides with the coordinated means of endosomal escape inherent to the LCP

formulation showed promise for improved capabilities in transfection overall. Through a fluorescence-based method, LCP was confirmed able to encapsulate plasmid DNA to roughly 50% efficiency; further, encapsulation was not affected by cocondensation with the peptides explored in formulation. Considering optimized (targeted and highly PEGylated) LCP, which contained no peptide, six hours after intravenous injection, a predominant amount of the injected dose was found to reside in the liver (~50%); further, on the intracellular level of the hepatocytes (Figure 7.4), fluorescently labeled DNA was found to be evenly distributed within the cytoplasm, implying effective hepatocyte delivery and endosomal escape, but poor nuclear localization. However, coencapsulation of such DNA with specific octaarginine peptides, which we refer to as monocyclic CR8C (mc-CR8C, a 10-mer composed of eight arginine residues flanked by two terminal cysteines, cyclized through a disulfide linkage) and linear CR8C (l-CR8C, the uncyclized form of the peptide), led not only to endosomal release, but a dramatic localization of the fluorescent signal in the nucleus by comparison. Further, the observed fluorescence distribution elicited by HD injection, currently the most powerful preclinical form of liver gene transfer (Liu, Song, & Liu, 1999), was diffuse and inconsistent across organ sections. Such results directly translated to transgene expression at 24 h postinjection; LCP coencapsulating

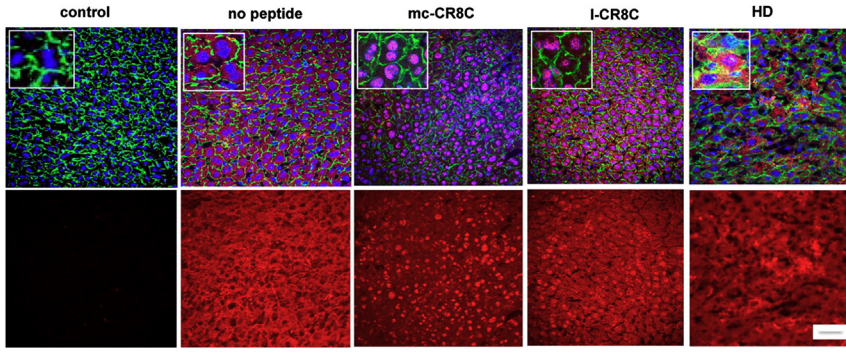

Figure 7.4 ***Intracellular Cy3-DNA fluorescence distribution in cryosections of liver tissues from C57BL/6 mice treated with LCP-(Control DNA)-Gal, LCP-(Cy3-DNA)-Gal, LCP-(Cy3-DNA + mc-CR8C)-Gal, LCP-(Cy3-DNA + l-CR8C)-Gal, and hydrodynamic (HD) injection 6 h after injection.*** Top images are fluorescence overlay of red from Cy3-DNA, blue from 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei, and green from fluorescein isothiocyanate(FITC)-labeled Phalloidin-stained actin; bottom images are Cy3-DNA fluorescence only. Inset images are an increased magnification, to better illustrate intranuclear Cy3-DNA fluorescence. Monocyclic abbreviated to mc, linear abbreviated to l. Scale bar: 25 μm. *Reprinted with permission from Hu et al. (2013).*

mc-CR8C elicited dramatic improvements in red fluorescent protein (RFP) transgene expression in hepatocytes (as compared to both no peptide and l-CR8C coencapsulation) and, on a quantifiable basis, improved luciferase transgene activity in the liver by roughly 10-fold over l-CR8C and over 200-fold over transgene delivery alone. Gene expression was relatively specific to the liver as well, despite significant accumulation in splenocytes on a %ID/gram basis (Hu et al., 2013). While the mechanism behind such profound increases remains uncertain, one could speculate as to the observed results through the strength of condensation which the peptides provide to the complex. Through a fluorescence-based method, l-CR8C was shown to more extensively condense DNA at experimental concentrations than mc-CR8C, and further limit release of DNA in the presence of competing anionic species (hyaluronan). It remains possible that, while both peptides support similar degrees of translocation of plasmid DNA into the nucleus, release of the vector upon translocation and subsequent gene expression may be more limited in the case of l-CR8C versus mc-CR8C (by comparison, hydrodynamically injected transgene should be relatively unimpeded in expression) (Hu et al., 2013).

To summarize thus far, we have highlighted the benefits of LCP in functionally addressing a considerable number of the commonly encountered barriers to effective drug delivery in general. The CaP nanoprecipitation process provides a readily amenable means by which to encapsulate a wide variety of drugs with desirable efficiency, as well as a stable DOPA coating, which can be easily functionalized with a variety of lipid-based materials to form an asymmetric lipid bilayer. High degrees of PEGylation made possible by the supported bilayer structure of LCP both elongate the kinetic elimination profile and alter the local cellular tropism for such nanoparticles in the liver. Active targeting strategies likely influence the global distribution to the liver, the rate of distribution to the liver, and the mechanism of cellular uptake of LCP. Further, the CaP cores that effectively entrap their cargo serve doubly as an acid-labile release mechanism, which, together with the cationic lipids on the liposome surface, serves to disrupt the endosome and release the cargo into the cytoplasm. Hereafter in the delivery process, however, functional improvements in transfection efficiency are less well explored on a mechanistic level. Nevertheless, we will describe the current state of the art in LCP customized for gene delivery, highlighting influences from the field, dramatic responses in efficacy, applications in a number of disease models, and future directions.

3. POTENTIAL THERAPEUTIC APPLICATIONS OF LCP: CANCER, CHRONIC LIVER DISEASE, AND GENE THERAPY

3.1 LCP as a Potent Vehicle for Treatment of Nonalcoholic Fatty Liver Disease

A variety of nanosystems have been developed for the purposes of treating common liver diseases, often showing profound efficacy. For example, acid-sensitive polyketal-based microparticles delivering siRNA against TNF-α were shown to effectively abrogate liver damage in a mouse acute liver failure model at dramatically low doses (3.5 μg/kg) (Lee et al., 2009). Further, with regard to the Dynamic PolyConjugate system described earlier, recent results suggest that coinjection of the polymer system with Chol-modified siRNA provides similar silencing efficacy as the conjugate itself, and that alternative systems of separate therapeutic and endosomolytic materials may be able to coordinate over a more temporally organized mode of delivery (i.e., separate injections of drug and polymer) (Wong et al., 2012; Wooddell et al., 2013). The following chapter describes in more detail the state of current hepatic transfection systems (Haynes & Huang, 2013), many of which can establish near-complete gene silencing at low μg/kg dosing levels; in this vein, we sought to employ the mechanistic potential of LCP in the treatment of a truly widespread condition.

With dramatic increases over the past half century in the Western world, particularly the United States, of obesity, the prevalence of a condition known as NAFLD can reach proportions as high as 30% (Starley, Calcagno, & Harrison, 2010). NAFLD is indicated in hallmark fashion through dramatic increases in hepatic steatosis (fat accumulation, often in large intracellular droplets) and serves as a precursor for a variety of hepatic complications such as liver cancer, hepatitis, cirrhosis, and liver failure; however, there are no currently approved medical therapies for NAFLD (Oz, Im, Chen, de Villiers, & McClain, 2006; Starley et al., 2010). Further, CD36, a hepatic transport protein that regulates free fatty acid uptake into and metabolism by the hepatocytes (Abumrad, Harmon, & Ibrahimi, 1998; Vannieuwenhoven et al., 1995), has been implicated in conditions of steatosis through increased expression and translocation to the plasma membrane (Koonen et al., 2007; Miquilena-Colina et al., 2011), while reductions in gene expression levels correlate to reductions in hepatic steatosis (Liu, Purushotham, Wendel, & Belury, 2007; Lopez-Parra et al., 2008).

To this end, we have shown that LCP could support the treatment of NAFLD, through the specific siRNA-mediated knockdown of CD36 protein

expression, in a relevant mouse model of the disease (Yunxia Hu, Matthew T. Haynes, Youngee Chung, Chin-Ying Chung, Yang Yang, Yuhua Wang, Chunshun Zhao, Feng Liu, Leaf Huang, unpublished data). It has been established that mice fed a diet deficient in methionine and choline (MCD diet) develop measurable hepatic steatosis in 1–3 weeks (Sahai, Malladi, Melin-Aldana, Green, & Whitington, 2004; Weltman, Farrell, & Liddle, 1996), and indeed, female C57/BL6 mice fed the MCD diet developed NAFLD after 15 days. Analyzed through a fluorescence method, LCP provided efficient means for oligonucleotide encapsulation and presented strong and diffuse fluorescent signal in the hepatocytes of the liver after intravenous injection, most notably in the ASGPR-targeted formulation over the untargeted formulation. Such efficient delivery was corroborated through treatment with the optimized LCP formulation delivering siRNA against CD36. Dramatic and significant reductions in CD36 protein expression levels, alanine transaminase levels, and macro- and microvesicular steatosis, both on a volumetric basis and a basis of total lipid droplet count, were observed by such means. Thus, the fatty acid transporter CD36 represents a worthwhile target in the treatment of NAFLD, while LCP optimized for hepatic accumulation and hepatocyte uptake proves to be a valuable RNAi system through which to silence CD36.

3.2 LCP as a Rational Vector for Enhanced Hepatic Transgene Expression

In terms of hepatic transgene expression, a variety of synthetic systems have shown satisfying results (Fortune et al., 2011; Gao, Yin, Chen, Zhang, & Li, 2011; Kim, Lei, Stolz, & Liu, 2007; Liu et al., 2011); however, the most efficacious system to date has been the Multifunctional Envelope-type Nano-Device (MEND) established by Harashima and colleagues (Khalil et al., 2011; Mudhakir & Harashima, 2009). Their nanoparticle system shares with ours an extensive appreciation toward multifunctionality. Their core coacervates of plasmid DNA and PEI shares great similarities to LPD (Li & Huang, 2009; Tan et al., 2002), providing a ready means of loading and stabilizing the therapeutic of interest within the lipid nanoparticle. The liposome itself, however, is functionalized in two important ways: an octaarginine peptide–lipid conjugate (STRR8/DOPE) promotes high accumulation of the nanoparticles in the liver, while a Chol conjugate with a pH-sensitive, fusogenic peptide (Chol-GALA) further promotes internalization and endosomal escape. In all, their outperformance of a wide variety of simpler synthetic systems (10^6 RLU/mg protein at 2.5 mg DNA/kg injected dose) serves to underscore the power of the multifunctional approach.

Noting the dramatic improvements made within the optimized LCP formulation, we sought to justify its utility to the field in comparison between formulations. By comparison to this most well-optimized R8-GALA-MEND system (Khalil et al., 2011), hepatic luciferase activity through mc-CR8C coencapsulation and delivery through ASGPR-targeted LCP (LCP-(mc-CR8C/pLuc)-GAL, 4.6×10^7 RLU/mg protein at 0.3 mg DNA/kg dose) outperforms this nanosystem to a notable degree (Hu et al., 2013). However, as one cannot be certain that measurements across laboratories are indeed directly comparable, relationships were sought between LCP and HD injection, a method developed by our collaborators and with which we have much experience (Liu et al., 1999). Though the optimized LCP formulation remains roughly 100-fold below the expression levels elicited via HD injection, such results represent a dramatic improvement in the state of the art in nonviral liver gene transfer (Hu et al., 2013). We are currently exploring potential therapeutic avenues, with high hopes for meaningful efficacy in the treatment of a variety of hepatic diseases.

3.3 LCP as a Combination Delivery System for Cancer Therapy

Lung cancer is the leading cause of death among all organ subcategories (Siegel, Ward, Brawley, & Jemal, 2011) and often presents as difficult to mediate surgically due to its advanced stage (ease of primary tumor resection, local and distant metastasis) upon diagnosis. Lacking in significant improvements in long-term survival rates through either surgery or chemotherapy (Molina, Adjei, & Jett, 2006), alternative treatment options remain highly sought after. With advances made in high-throughput chemical synthesis, vast libraries of compounds for delivery in RNA interference in particular, termed "lipidoids," along with others, have helped to set the bar for optimal *in vivo* efficacy well into the μg/kg dosing range, allowing for the potential of vast degrees of combination RNAi strategies targeting a variety of cell signaling processes for expression silencing as well (Akinc et al., 2008; Lee et al., 2009; Love et al., 2010). Though the strongest potency with such materials lay in the liver, low-mg/kg therapeutic efficacy has been shown by means of local (Goldberg et al., 2011; Kim, Jeong, Lee, Kim, & Park, 2008; Place et al., 2012) and systemic (Hu-Lieskovan, Heidel, Bartlett, Davis, & Triche, 2005; Kim et al., 2008) delivery for cancer therapy. With simple means for encapsulation and release, along with dynamic levels of PEGylation potential and active targeting, LCP provides reliable means for low-dose silencing and was explored in various models of lung cancer in this regard. In a simple A549 subcutaneous

tumor model, systemic administration of LCP, coformulated with separate sequence-specific siRNA (against MDM2, c-myc, and VEGF) and targeted via anisamide, successfully inhibited tumor growth with an ED_{50} at sub-mg/kg dosing levels (200 μg/kg) (Yang, Hu, et al., 2012), a dramatic improvement over alternative formulations (Figure 7.5). Given the flexibilities in encapsulation via CaP, combination therapies with phosphorylated small-molecule prodrugs, which possess notoriously rapid blood clearance (e.g., gemcitabine monophosphate, GMP) are readily accessible as well. As described in Chapter 4, combination siRNA/GMP delivery via LCP has exhibited similarly strong efficacy at such dose levels as well in both subcutaneous H460 and orthotopic A549 lung cancer models, with the combination siRNA approaches outperforming encapsulated drug alone in both cases (Zhang, Peng, Mumper, & Huang, 2013; Zhang et al., 2013). Further, in a syngeneic model of melanoma (B16F10) metastasis to the lung, metastasis growth was dramatically inhibited and mean survival was

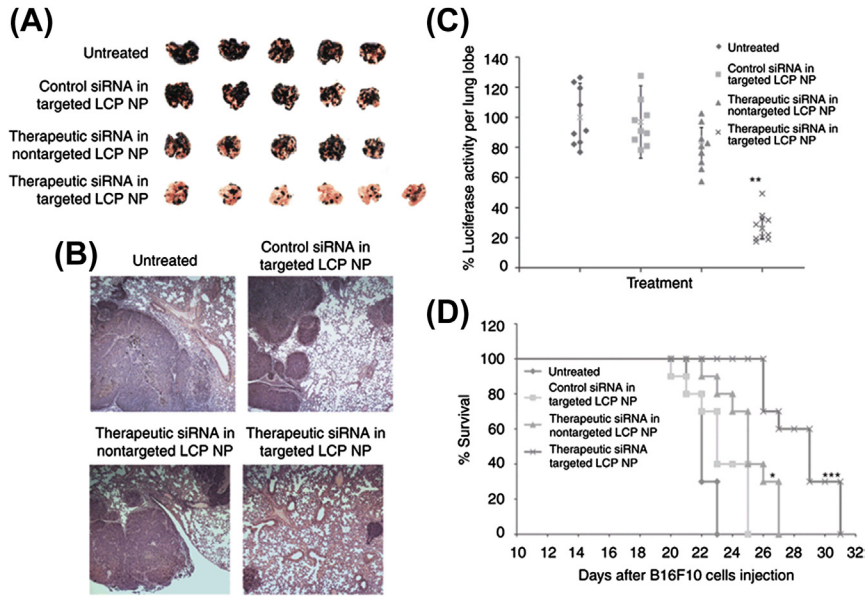

Figure 7.5 ***Antimetastasis efficacy of therapeutic siRNA delivered by LCP nanoparticles.*** (A) Photographs of lungs excised from tumor-bearing mice 19 days after injection (intravenous administration of B16F10 cells) and four nanoparticle treatments. (B) Quantification of lung metastasis by luciferase activity on day 19. (C) Images of hematoxylin and eosin (H&E) staining of the lung tissue sections (40× mag.). (D) Survival analysis of B16F10 lung metastases-bearing mice after four treatments. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$. *Adapted with permission from Yang, Li, et al. (2012).*

enhanced through the pooled siRNA combination described above, again at sub-mg/kg dosing (360 μg/kg) (Yang, Li, Liu, & Huang, 2012). In these manners, LCP represents a formulation with profound potential for systemically mediated cancer therapy.

4. LCP NANOPARTICLES: CONCLUSIONS

Though significant advances have been made in maximizing the strengths of more unilaterally focused systems, we share the perspective that a multifunctional approach such as LCP will inevitably provide the flexibility in optimization necessary to make profound advances in the clinic with regard to nonviral gene delivery systems. Through a variety of mechanistic explorations and disease models, we have shown the power of such considerations through our LCP platform technology and look forward to future advances in dealing with the biological complexities which these diseases present.

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CHAPTER EIGHT

Polymers for Nucleic Acid Transfer—An Overview

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Contents

Abstract

For the last five decades cationic polymers have been used for nucleic acids transfection. Our understanding of polymer—nucleic acid interactions and their rational use in delivery has continuously increased. The great improvements in macromolecular chemistry and the recognition of distinct biological extra- and intracellular delivery hurdles triggered several breakthrough developments, including the discovery of natural and synthetic polycations for compaction of nucleic acids into stable nanoparticles termed polyplexes; the incorporation of targeting ligands and surface-shielding of polyplexes to enable receptor-mediated gene delivery into defined target tissues; and strongly improved intracellular transfer efficacy by better endosomal escape of vesicle-trapped polyplexes into the cytosol. These experiences triggered the development of second-generation polymers with more dynamic properties, such as endosomal pH-responsive release mechanisms, or biodegradable units for improved biocompatibility and intracellular release of the nucleic acid pay load. Despite a better biological understanding, significant challenges such as efficient nuclear delivery and persistence of gene expression persist. The therapeutic perspectives widened from pDNA-based gene therapy to application of novel therapeutic nucleic acids including mRNA, siRNA, and microRNA. The finding that different therapeutic pay loads require different tailor-made carriers complicates preclinical developments. Convincing evidence of medical efficacy still remains to be demonstrated. Bioinspired multifunctional polyplexes resembling "synthetic viruses" appear as attractive opportunity, but provide additional challenges: how to identify optimum combinations of functional delivery units, and how to prepare such polyplexes reproducibly in precise form? Design of sequence-defined polymers, screening of combinatorial polymer and polyplex libraries are tools for further chemical evolution of polyplexes.

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1. FIVE DECADES IN POLYPLEXES: CHALLENGES AND BREAKTHROUGHS

The history of nucleic acid transfection by polymers comprises already five decades. The infectivity of isolated phenol-extracted single-stranded poliovirus RNA was up to 100-fold enhanced by formulation with cationic proteins (Smull & Ludwig, 1962) and polymers such as DEAE dextran (Vaheri & Pagano, 1965). Analogous findings were made for transfer of SV40 viral DNA, enhancing infectivity up to 100,000 times (McCutchan & Pagano, 1968). Vaheri and Pagano suggested a stabilizing DEAE dextran coating of RNA as mechanism, additional possibilities including interaction of the polycation with cell surface, because pretreatment of cells with DEAE dextran also showed some effect. Since then our understanding of polymers and their rational use in gene delivery have gradually increased. This includes the transfection of complexes of polyornithine with exogenous DNA (Farber, Melnick, & Butel, 1975), and the finding that polyamines such as polylysine not only bind but also condense nucleic acids into compact protected structures (Chattoraj, Gosule, & Schellman, 1978; Laemmli, 1975). The work by George and Catherine Wu, reporting receptor-mediated gene transfer (Wu & Wu, 1987, 1988), can be considered as a conceptional breakthrough in the development of polyplexes, i.e., nucleic acid polymer complexes (Felgner et al., 1997). Wu & Wu first synthesized defined biochemical conjugates of asialoorosomucoid (ASOR) and poly(L) lysine (Figure 8.1(A) and (B)). They used these conjugates for DNA polyplex formation by applying a protocol analogous as used in nucleosome reconstitutions; polyplexes were formed in a thermodynamically controlled process, mixing DNA and ASOR-polylysine at high salt (2 M), followed by gradual reduction of the salt concentration to physiological 150 mM by dialysis, and finally filtering polyplexes through a 0.45 μm membrane. The hepatocyte-specific asialoglycoprotein receptor preferentially recognizes three terminal galactose molecules presented by triantennary carbohydrates of desialinated glycoproteins. Applying the ASOR-polylysine polyplexes, Wu & Wu were able to demonstrate asialoglycoprotein receptor-mediated gene transfer both in vitro into hepatocytic cells and also in vivo upon intravenous administration, resulting in significant liver-specific gene transfer. Other groups successfully extended the concept to other receptor-ligand systems for targeting other tissues (Ferkol, Kaetzel, & Davis, 1993) or tumors (Wagner, Zenke, Cotten, Beug, & Birnstiel, 1990).

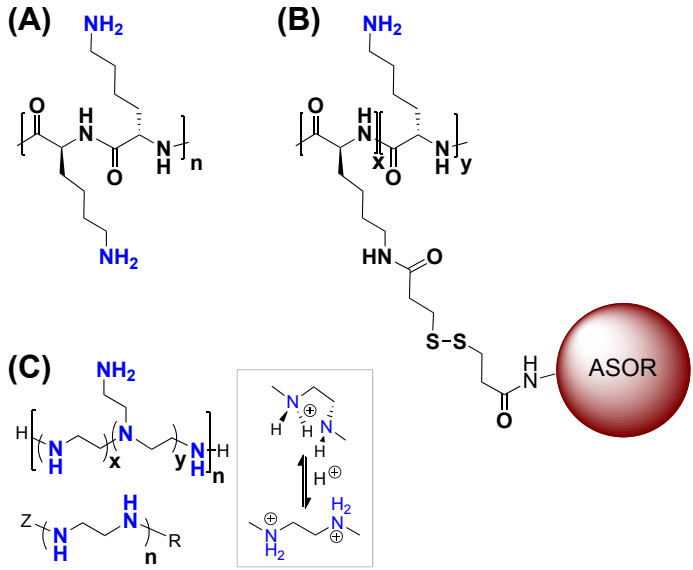

Figure 8.1 ***First-generation polycations and conjugates.*** (A) Poly(L)lysine (pLys). (B) Asialoorosomucoid-pLys conjugates via disulfide linkage (Wu & Wu, 1987). (C) Polyethylenimine (PEI) in branched or linear form (Boussif et al., 1995), presented with different degrees of protonation.

Transfection efficiencies of polylysine-based targeted polyplexes (Zauner, Ogris, & Wagner, 1998) however remained moderate until the discovery of new agents, such as chloroquine, which stimulate endosomal burst and strongly enhance the gene delivery (Cotten et al., 1990). The presence of noninfectious viral particles such as inactivated adenoviruses (Cotten, Wagner, Zatloukal, & Birnstiel, 1993; Curiel, Agarwal, Wagner, & Cotten, 1991) or rhinovirus particles (Zauner, Blaas, Kuechler, & Wagner, 1995) in the transfection medium-enhanced efficiency of receptor-targeted polyplexes up to more than 1000-fold. The added viruses possess specific endosomal membrane-disruption domains. When entering via their own receptor-mediated endocytosis route, they meet internalized polyplexes and also facilitate the cytosolic release of these nanoparticles. To ensure the colocalization, the strategy was refined by direct coupling polyplexes with viral particles (Curiel et al., 1992; Wagner, Zatloukal, et al., 1992). In addition, instead of whole virus particles, synthetic virus-derived or artificial membrane-active peptides (Plank, Zauner, & Wagner, 1998; Wagner, 1998; Wagner, Plank, Zatloukal, Cotten, & Birnstiel, 1992) were incorporated.

Acidification of endosomes by the vacuolar ATP-dependent V-ATPase is a key trigger for the mentioned viral and artificial membrane-disruption processes. For example, the endosomal protonation is required for endolysosomal entrapment of the weakly basic drug chloroquine, which results in accumulation of the protonated drug and osmotic swelling of endolysosomes. Based on rational considerations, Jean-Paul Behr and collaborators in their search for transfection agents screened cationic polymers with proton sponge characteristics; such proton sponges would only be partly protonated at neutral extracellular pH sufficient for nucleic acid binding, but would increase their protonation and charge density within endosomes. Influx of chloride counterions and water should osmotically destabilize the polyplex-containing endosomal vesicle. This nice and hypothesis-driven approach resulted in the discovery of a very potent transfection polymer, polyethylenimine (PEI; Figure 8.1(C)) (Behr, 1997; Boussif et al., 1995; Coll et al., 1999; Zou, Erbacher, Remy, & Behr, 2000). Because of its chemical simplicity and stability, its high efficiency both in vitro and in vivo with a moderate cytotoxicity, PEI has been broadly used as a transfection agent and also been tested in clinical studies in localized applications. Details are presented in the following chapter (Neuberg & Kichler, 2014). Although the detailed endosomal escape mechanism of PEI-type proton sponges (Benjaminsen, Mattebjerg, Henriksen, Moghimi, & Andresen, 2013; Miyata, Nishiyama, & Kataoka, 2012; Wagner, 2012) is still under discussion, significant evidence for the original hypothesis was established by several investigators (Akinc, Thomas, Klibanov, & Langer, 2005; Boeckle et al., 2004; Kichler, Leborgne, Coeytaux, & Danos, 2001; Ogris et al., 1998; Sonawane, Szoka, & Verkman, 2003; Wightman et al., 2001).

As outlined in the following sections and various reviews (Brown, Schatzlein, & Uchegbu, 2001; Dang & Leong, 2006; De Smedt, Demeester, & Hennink, 2000; Itaka & Kataoka, 2009; Midoux, Breuzard, Gomez, & Pichon, 2008; Pack, Hoffman, Pun, & Stayton, 2005; Park, Jeong, & Kim, 2006; Schaffert & Wagner, 2008; Schatzlein, 2003; Wagner, 2004, 2007; Wagner & Kloeckner, 2006), further polyplex development was pursued in at least two distinct directions in parallel. On the one hand, greatly encouraged by the discovery of PEI, the quest for even better cationic polymers for forming the polyplex core brought conceptual breakthroughs such as design of biodegradable polymers (Han, Mahato, Sung, & Kim, 2000; Lim et al., 2000), screening of combinatorial libraries (Akinc, Lynn, Anderson, & Langer, 2003; Anderson, Lynn, & Langer, 2003), and precise sequence-defined polymers (Hartmann & Börner, 2009; Schaffert, Troiber, et al., 2011;

Wang, Jensen, & Lu, 2007). On the other hand, decoration of polyplex cores with novel targeting, shielding, and intracellular delivery functions resulted in the first prototypes of bioinspired synthetic viruses (Miyata et al., 2012; Murthy, Campbell, Fausto, Hoffman, & Stayton, 2003a; Plank, Zatloukal, Cotten, Mechtler, & Wagner, 1992; Wagner, Plank, et al., 1992; Zuber, Dauty, Nothisen, Belguise, & Behr, 2001).

2. OPTIMIZING THE CORE: BIODEGRADABLE AND BIOCOMPATIBLE POLYMERS

Three aims were dominating the search: (1) identify polymers with a higher potency in intracellular delivery; (2) improve biocompatibility; and (3) enhance pharmaceutical precision. A key lesson learned from understanding the mechanism of PEI polyplexes has been the fact that polyplexes must not be static stable structures, but have to be dynamically responsive during the delivery process (Wagner, 2007). Thus, polymers ideally should change their properties with time and location, to stably pack nucleic acid outside the cell, facilitate the transfer across the endosomal barrier triggered by acidification, and support intracellular release and bioavailability of the therapeutic nucleic acid (DNA, mRNA, siRNA) in the required cellular compartment. Not surprisingly, requirements differ between DNA and siRNA (Kwok & Hart, 2011; Scholz & Wagner, 2012).

PEI, due to its proton sponge characteristics, is a dynamic polymer, but nevertheless cannot fulfill all delivery tasks in an optimum fashion. This is well illustrated by the finding that DNA polyplexes formed with branched PEI are more stable than linear PEI polyplexes, but linear PEI polyplexes were found to be more effective in gene transfer; apparently intracellular release (Itaka et al., 2004) and also nuclear entry of DNA (Brunner, Furtbauer, Sauer, Kursa, & Wagner, 2002) can be better managed by the less-stable linear PEI polyplexes. Despite many favorable properties, PEI displays also drawbacks: it is nondegradable and significantly toxic (Grandinetti, Ingle, & Reineke, 2011; Moghimi et al., 2005) in a molecular weight-dependent manner. Cytotoxicity includes cell surface and organelle membrane (mitochondria, nucleus) defects, triggering apoptosis, necrosis, and also block of ATP synthesis (Hall et al., 2013). Beyond direct cytotoxicity, PEI like many other polycations interacts with the innate immune system and triggers complement activation (Plank, Mechtler, Szoka, & Wagner, 1996) in vitro and in vivo, resulting, for example, in anaphylactic effects in pigs upon intravenous administration (Merkel et al., 2011).

For increased biocompatibility and biodegradability, nature-derived polymers (Dang & Leong, 2006) such as based on chitosan (poly-D-glucosamin, generated by deacetylation of chitin) derivatives (Chang, Higuchi, Kawakami, Yamashita, & Hashida, 2010; Erbacher, Zou, Bettinger, Steffan, & Remy, 1998; Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008; Howard et al., 2006; Koping-Hoggard et al., 2001; Lee et al., 2007; MacLaughlin et al., 1998; Mansouri et al., 2004; Park et al., 2001; Sato, Ishii, & Okahata, 2001; Thanou, Florea, Geldof, Junginger, & Borchard, 2002; Yu, Wang, Chen, Deng, & Jing, 2006), cationic cyclodextrin conjugates (Arima, Kihara, Hirayama, & Uekama, 2001; Bellocq, Pun, Jensen, & Davis, 2003; Cryan, Holohan, Donohue, Darcy, & O'Driscoll, 2004; Davis, 2009; Davis et al., 2010; Hwang, Bellocq, & Davis, 2001; Ping, Hu, Tang, & Li, 2013; Pun et al., 2004), or cationized collagens (Takeshita et al., 2005; Young, Wong, Tabata, & Mikos, 2005) were evaluated as DNA or siRNA carriers.

Alternatively, motivated by the knowledge that cytotoxicity of polycations such as polylysine and PEI was largely molecular weight dependent, biodegradable polymer analogs were designed. Hydrolyzable bonds (esters, acetals, Schiff bases) and bioreducible disulfide linkages were introduced, as reviewed in Wagner & Kloeckner (2006) and Yu & Wagner (2009). Pioneering work by the laboratory of Sung Wan Kim (Han et al., 2000) evaluated a series of strategies; for example, they replaced the amide bonds of polylysine by ester bonds resulted in the biodegradable and nontoxic analog poly(alpha-(4-aminobutyl)-L-glycolic acid) (Figure 8.2(A)). However, like for polylysine only modest transfection activity was obtained, probably because of the lack of efficient endosomal escape functionality (Lim et al., 2000). More effective transfection polymers were generated by synthesizing hyperbranched network-type poly (amino esters) (Lim, Kim, Suh, & Park, 2002) (Figure 8.2(B)) and poly (β-amino esters) by Michael addition of amino-containing molecules with acrylate esters (Akinc, Anderson, Lynn, & Langer, 2003; Forrest, Koerber, & Pack, 2003; Lim et al., 2002). A conceptual milestone in search of better transfection polymers was set by laboratory of Robert Langer. In a combinatorial approach based on Michael addition of various primary amines or secondary diamines with a variety of bis-acrylates (Figure 8.2(C)), they synthesized 2350 degradable cationic polymers and performed a large-scale transfection screen, identifying candidates with interesting gene transfer properties (Akinc, Lynn, et al., 2003; Anderson, Akinc, Hossain, & Langer, 2005; Anderson et al., 2003). This approach was extended to additional chemistries and the incorporation of aliphatic hydrocarbon residues, resulting in a series of hydrophobic oligocationic

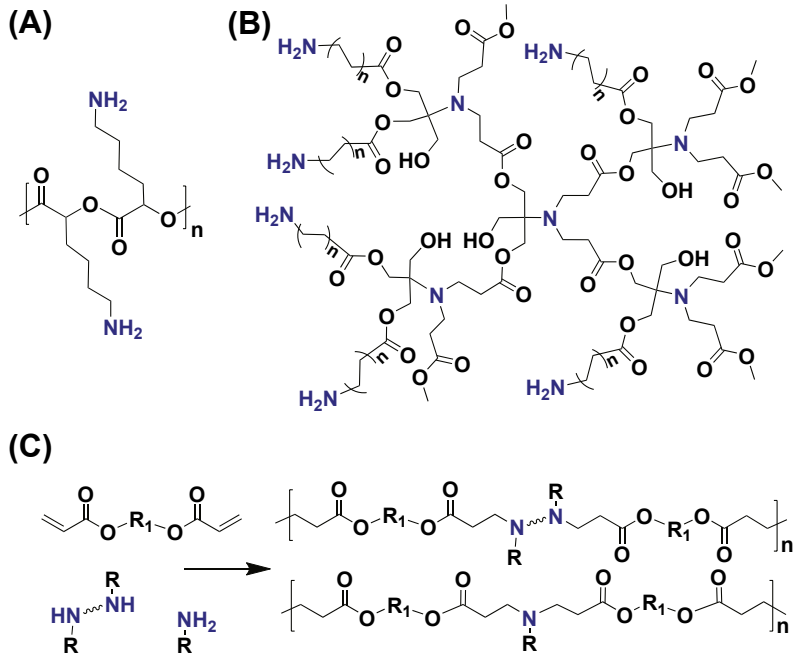

Figure 8.2 ***Biodegradable poly (amino esters).*** (A) Poly(alpha-(4-aminobutyl)-L-glycolic acid) (Lim et al., 2000). (B) Network-type hyperbranched poly (amino ester) (Lim et al., 2002). (C) Libraries of beta-aminoester-based polymers generated by Michael addition (Anderson et al., 2003; Lynn et al., 2001).

compounds (lipidoids) including highly potent DNA and siRNA delivery agents (see chapter by Dahlman, Kauffman, Langer, & Anderson, 2014).

The groups of Feijen, Engbersen, and Kim extended the Michael addition strategy to include bioreducible disulfide block-containing acrylates (Figure 8.3(A)); as amine reactants they also included small oligoethylenimine building blocks including triethylene tetramine, yielding bioreducible and effective analogs of PEI (Lin et al., 2008; Christensen et al., 2006; Hoon et al., 2007; Kim & Kim, 2011). Lu and colleagues generated bioreducible disulfide polymers by a different route (Figure 8.3(B)), starting with solid-phase-synthesized peptides containing triethylene tetramine, histidines, and disulfide-forming cysteines (Wang et al., 2007).

Earlier, alternative approaches to more biocompatible PEI analogs started by cross-linking low molecular weight PEI (0.6–1.8 kDa oligoethylenimines) into structures of typical PEI size of 20–50 kDa. Pack and colleagues synthesized degradable PEI derivatives with low toxicity and efficient gene transfer activity up to 16-fold higher than for 25-kDa branched

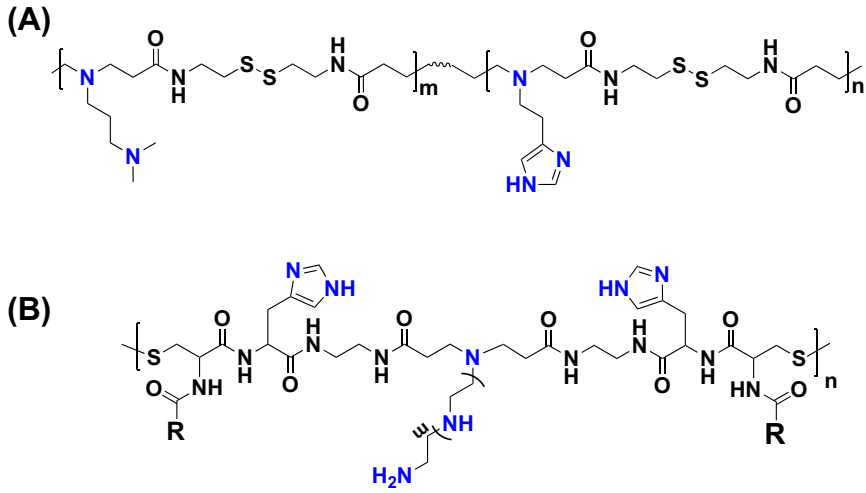

Figure 8.3 ***Bioreducible disulfide-based polymers.*** (A) Polymers generated by Michael addition reaction (Lin et al., 2007). (B) Polymers generated from solid-phase-synthesized bis-cysteine oligomers by oxidation (Wang et al., 2007).

PEI by cross-linking 0.6-kDa PEI with diacrylates of either 1,3-butanediol or 1,6-hexanediol, yielding 14–30 kDa polymers (Forrest et al., 2003) (Figure 8.4(A)).Analogous cross-linking via disulfide bridges was reported by Gosselin and colleagues (Gosselin, Guo, & Lee, 2001) using PEI of 0.8 kDa and the bifunctional cross-linkers dithiobis(succinimidylpropionate) (DSP) or dimethyl-3-3′-dithiobispropionimidate (DTBP). Using DSP and DTBP, the primary amino groups of PEI which are cross-linked were converted into neutral amide or cationic amidine groups, respectively (Figure 8.4(B)). Also for linear PEIs, introduction of disulfide bonds into the polymer backbone greatly reduced cytotoxicity but retained efficacy (Breunig, Lungwitz, Liebl, & Goepferich, 2007; Lee et al., 2005).Variation of reaction conditions can be used to tune physicochemical and biological properties of these ester- and disulfide-based analogs (Kloeckner, Bruzzano, Ogris, & Wagner, 2006; Kloeckner, Wagner, & Ogris, 2006; Yu, Russ, & Wagner, 2009) used in DNA and also siRNA transfections (Tarcha et al., 2007; Tietze et al., 2008).

Besides hydrolyzable ester and bioreducible disulfide bonds, pH-sensitive linkages were also incorporated into polymer backbones. In a proof-of-concept study, Kim and colleagues cross-linked low molecular weight PEI by glutardialdehyde under Schiff base formation, resulting in a hydrolyzable transfection carrier with efficiency similar to that of standard PEI (Kim, Park, Lee, Park, & Kim, 2005). Ketal bond-bearing

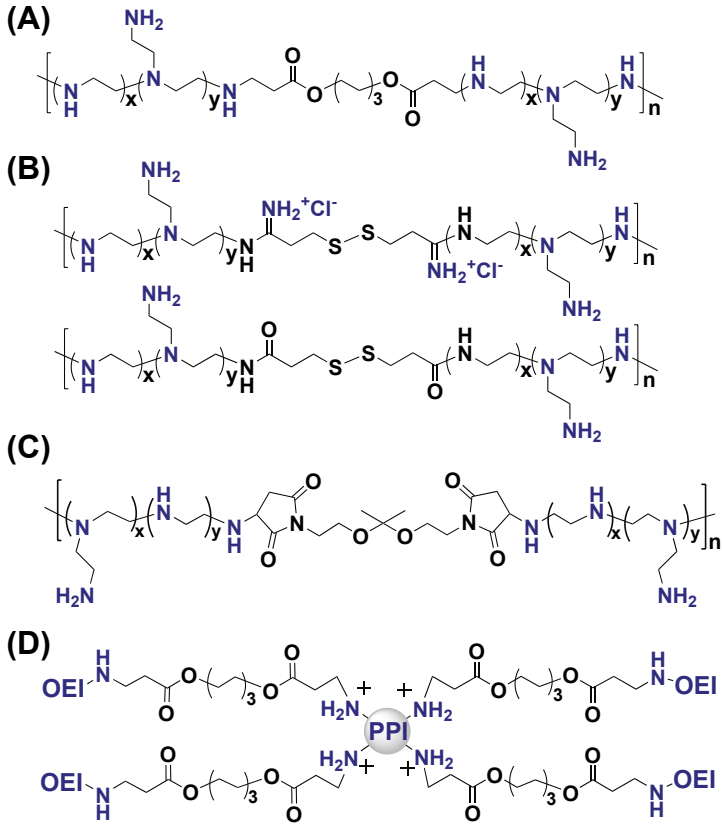

Figure 8.4 ***Degradable polymer conjugates based in low molecular weight oligoethylenimine (OEI).*** Oligoethylenimine cross-linked (A) via ester (Forrest et al., 2003), (B) via disulfide (Gosselin et al., 2001), or (C) via acetal bonds (Knorr, Russ, et al., 2008). (D) Pseudodendritic linkage via ester bond to core polypropylenimine (PPI) dendrimer (Russ, Gunther, et al., 2008).

PEI analogs were also developed by Knorr et al. (Figure 8.4(C)); polyplexes should dissociate efficiently because of the hydrolysis of the ketal backbone bond in the endosomal acidic environment. In fact, these acid-sensitive polymers displayed better gene transfer ability and improved biocompatibility compared to their acid-stable controls (Knorr, Ogris, & Wagner, 2008; Knorr, Russ, Allmendinger, Ogris, & Wagner, 2008). Also acid-degradable methacrylamide DNA nanoparticles were designed, which under endosomal acidic conditions release the cationic groups from the polymer backbone, resulting in release and transfection of the DNA (Ko, Ziady, Lu, & Kwon, 2008).

Several of the described biocompatible polymer preparations suffer from a new problem: polymer conjugates usually represent heterogenous mixtures in macromolecule sizes, topologies, and chemical conjugation sites. As a both structurally aesthetic and pharmaceutically attractive solution, dendrimers have been introduced. For gene transfer (Dufès, Uchegbu, & Schätzlein, 2005), nondegradable dendrimers based on polyamidoamine "Starburst" dendrimer with not only proton sponge and high transfection activity but also significant cytotoxicity (Choi et al., 2004; Haensler & Szoka, 1993; Kukowska-Latallo et al., 1996, 2000; Tang & Szoka, 1997) were used. Also dendritic polylysines (Kawano, Okuda, Aoyagi, & Niidome, 2004; Luo et al., 2011; Ohsaki et al., 2002; Okuda, Sugiyama, Niidome, & Aoyagi, 2004), and different generations of polypropylenimine (PPI) dendrimers have been evaluated for gene delivery. Cell cytotoxicity was largely generation dependent; the generation 2 PPI dendrimer with eight arms combined sufficient DNA binding with a low level of cytotoxicity and a suitable in vitro gene transfer activity (Zinselmeyer, Mackay, Schatzlein, & Uchegbu, 2002), generation 3 derivatives a better suitable in vivo efficacy (Schatzlein et al., 2005). Incorporation of transferrin as large and biocompatible targeting ligand resulted in very encouraging activity in tumor mouse models (Dufes et al., 2005; Koppu et al., 2010).

Several efforts have been reported in combining the dendritic concept with the design of biodegradable polymers. For example, Haag and colleagues utilized dendritic oligoglycerol cores with oligoamine shells for siRNA transfection (Fischer et al., 2010). Russ and colleagues prepared pseudodendrimers consisting of a 0.8-kDa oligoethylenimine or PPI dendrimer core and a degradable hexanediol diacrylate ester shell, which was subsequently modified by various oligoamines, including oligoethylenimine (Figure 8.4(D)). Such pseudodendritic structures were small (below 5 kDa), better defined than randomly cross-linked polymer preparations (Frohlich, Edinger, Russ, & Wagner, 2012; Klutz et al., 2009; Russ, Elfberg, et al., 2008; Russ et al., 2010; Russ, Gunther, Halama, Ogris, & Wagner, 2008). They exhibited low cytotoxicity and good biodegradability with a half-life of 3 days under physiological conditions. Improved biocompatibility over standard PEI was also demonstrated by absence of erythrocyte aggregation ability. Intravenous administration of these novel structures in tumor-bearing mice could efficiently transfer DNA into subcutaneous tumors with low cytotoxicity, and lead to promising gene expression levels.

In parallel with the quest for novel, more potent, and biocompatible polymers, the increased understanding about the involved transfection

mechanisms triggered the modification of well-established transfection polymers. Along these lines, incorporation of hydrophilic polyethylene glycol (PEG) molecules (Ahn, Chae, Bae, & Kim, 2002) and/or carbohydrates (Erbacher, Roche, Monsigny, & Midoux, 1995; Pun et al., 2004; Reineke & Davis, 2003) greatly improved the biocompatibility of cationic polymers. Incorporation of histidine residues, which improve endosomolytic proton sponge characteristics, was shown to greatly improve the transfection efficacy of polylysine and PEI (Bertrand et al., 2011; Gomez, Pichon, & Midoux, 2013; Goncalves, Pichon, Guerin, & Midoux, 2002; Midoux & Monsigny, 1999). The efficacy of PEI for siRNA is limited; simple modifications of 25 kDa branched PEI, such as coupling of approximately 20% of nitrogens with tyrosine residues (Creusat & Zuber, 2008; Creusat et al., 2010), or 10% succinylation (Zintchenko, Philipp, Dehshahri, & Wagner, 2008), or pyridylthiourea grafting (Creusat et al., 2012), converted this PEI molecule into a potent siRNA transfection agent. In conclusion, several ways have been pursued to identify better transfection polymers, including use of nature-derived macromolecules, designing and screening novel biodegradable polymers, and refinement of existing transfection agents. As a note of caution, one always has to keep in mind that the proper selection of a core polymer is only one important part of polyplex design, but tuning by proper modifications and assembly with a polyplex shell is equally important.

3. OPTIMIZING THE SHELL: BIOINSPIRED SMART POLYPLEXES

For efficient extracellular and intracellular transfer of therapeutic nucleic acids, numerous distinct delivery functions have to be included, such as stabilization in the blood circulation, targeted cell binding and uptake, endosomal release, followed by delivery into the required cellular compartment in a bioactive form. The search for polymers and block copolymers, which inherently fulfill more than one delivery function, provided some advance. For example, PEG-PEI copolymers compact DNA by cationic charge interaction, partly shield the formed complexes by PEG domains, facilitate cell binding and uptake by residual cationic charges, and to some extent facilitate endosomal escape by additional cationization of PEI (Ahn et al., 2002). Nevertheless, such a polymer cannot efficiently fulfill all the delivery tasks. In addition, some of the delivery aims, such as optimum polyplex shielding as opposed to optimum endosomal release, may actually work against each other (Walker et al., 2005).

Inspired by the structure and function of natural viruses, which contain distinct functional subdomains within the viral particle for facilitating the various delivery steps, first prototypes of "synthetic viruses" were designed (Miyata et al., 2012; Murthy et al., 2003a; Plank et al., 1992; Wagner, Plank, et al., 1992; Zuber et al., 2001). The conceptual difference and advance over previous standard polyplexes were as follows: (1) design of tailor-made functional delivery domains and incorporation at different locations (core or shell) of the nanoparticle; (2) timely and spacially programmed activation of the delivery domains only at the biological cellular site where they are required. The latter characteristics require the incorporation of chemical sensing units, which are able to react to the specific biological microenvironment by conformational and physical changes and/or cleavage of chemical bonds. In this way, programmed, dynamic nanoparticles should be constructed (Wagner, 2007).

Numerous cell-targeting ligands have been successfully incorporated into polyplexes, either by direct conjugation to a core-forming polymer, see for example (Kircheis et al., 1997), by attachment to preformed polyplexes (Blessing, Kursa, Holzhauser, Kircheis, & Wagner, 2001), or as separate conjugates which are co-integrated into formed polyplexes (Kursa et al., 2003). The list of possible ligands contains large proteins such as transferrin and antibodies, peptides, vitamins, carbohydrates, and chemical ligands. Target organs and tissues which were successfully targeted by polyplexes in experimental models include the liver, lung, brain, and tumors. One of the most commonly used ligand, transferrin, was already used in clinical studies. In the 1990s, transferrin was applied as ligand in the very first polymer-based human gene therapy with an interleukin-2 gene-modified cancer vaccine (Schreiber et al., 1999) because of effective transferrin receptor targeting of primary melanoma cell cultures ex vivo. In a recent human clinical in vivo study, it was used for targeting PEGylated siRNA/cationized cyclodextrin polyplexes to tumors (Davis, 2009; Davis et al., 2010). For more information on various evaluated targeting ligands, the reader is referred to previous reviews (Wagner, 2012; Ogris & Wagner, 2011; Wagner, Culmsee, & Boeckle, 2005).

Frequently, especially when smaller ligands were applied, targeting ligands were incorporated together with shielding molecules such as PEG. This was based on early findings that polycationic carriers such as polylysine interact with the innate immune system and trigger complement activation (Plank et al., 1996). In addition, nonshielded cationic polyplexes were found to ionically interact and aggregate with serum and blood cells, which results in toxicity in vivo and loss of targeting ability. Pre- or postintegration of hydrophilic polymers such as PEG (Blessing et al., 2001;

Merdan et al., 2005; Pun et al., 2004; Walker et al., 2005), poly(hydroxypropyl methacrylate) (Carlisle et al., 2004; Fisher et al., 2000), hydroxyl ethyl starch (Noga et al., 2012), or hyaluronic acid (Hornof, de la, Hallikainen, Tammi, & Urtti, 2008; Ito, Yoshihara, Hamada, & Koyama, 2010) for polyplex surface modification have resolved the biocompatibility concerns. PEG-pLys DNA polyplexes were the first ones that were evaluated in a human clinical in vivo study (Davis & Cooper, 2007; Konstan et al., 2004; Walsh et al., 2006) in epithelia of cystic fibrosis patients.

Polyplex shielding from unspecific interactions however also reduces efficacy in transfecting target cells. In this respect, PEI polyplexes with acid-triggered PEG deshielding capabilities were found to have dramatically higher gene transfection efficiency both in vitro and in vivo compared to stable shielded polyplexes (Walker et al., 2005). The findings were explained by the hypothesis that a stable PEG shield would prevent interaction of the protonated cationic PEI with the endosomal vesicle membrane, whereas removal of PEG would overcome this problem (Figure 8.5(A)). Endosomal pH-sensitive bonds between polycations and PEG (Meyer & Wagner, 2006) used in this strategy included pyridylhydrazones (Fella, Walker, Ogris, & Wagner, 2008; Walker et al., 2005), acetals (Knorr, Allmendinger, Walker, Paintner, & Wagner, 2007; Knorr, Ogris, et al., 2008; Murthy et al., 2003a; Murthy, Campbell, Fausto, Hoffman, & Stayton, 2003b), and dialkylmaleic acid monoamides (Rozema et al., 2007; Wolff & Rozema, 2008). With the latter strategy, "Dynamic PolyConjugates" were designed for the asiologly-coreceptor-targeted siRNA delivery into the liver.

As reviewed by (Wolff & Rozema, 2008), a virus-like character requires that polyplexes become chemically dynamic (Wagner, 2008). In their work, endosomal acidification not only removes the hydrophilic shield from the nanoparticle, but also exposes hydrophobic lytic polymer domains which promote the release into the hepatocyte cytosol. Analogously, Meyer and colleagues (Martin Meyer et al., 2009; Meyer, Philipp, Oskuee, Schmidt, & Wagner, 2008) reported conversion of inactive polylysine polyplexes into highly active siRNA transfection agents by PEGylation and incorporation of endosomally activated lytic melittin peptides (Figure 8.5(B)).

Besides the acidic pH of endosomes, also the bio-reducing environment of the cytosol has been utilized for activation of delivery functions (Choi & Lee, 2008; Klein & Wagner, 2014; Saito, Amidon, & Lee, 2003) including the intracellular release of nucleic acids by reduction of disulfide-containing carrier polymers (McKenzie, Kwok, & Rice, 2000; Read et al., 2003). It has to be kept in mind that, in spite of a superior knowledge of intracellular

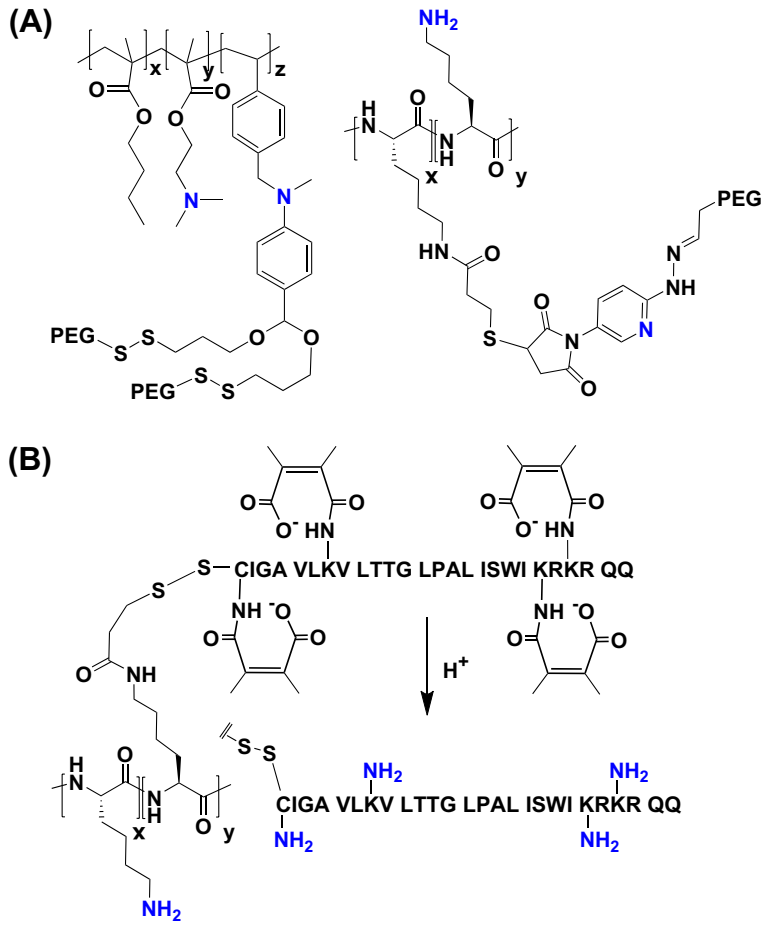

Figure 8.5 ***Dynamic polymer conjugates.*** (A) Endosomal pH-cleavable PEG-polycation conjugates with acetal (left, (Murthy et al., 2003b)) or pyridyl hydrazone (right, (Walker et al., 2005)) bonds. (B) pH-triggered unmasking of dimethylmaleic acid-protected lytic domains such as a melittin peptide sequence, amino acids presented as one letter code (Meyer et al., 2008).

biological processes, significant challenges such as inefficient nuclear delivery of DNA and persistence of subsequent gene expression persist. Proper nuclear delivery domains, although tested for more than a decade (Zanta, Belguise, & Behr, 1999; van der Aa et al., 2005), still remain to be validated (Remaut, Symens, Lucas, Demeester, & De Smedt, 2014).

In sum, introduction of additional functional domains in several cases tremendously improved efficacy of polyplexes. However, like in car manufacturing, where an engine can only work effectively if well combined with

the right power transmission and frame of a car, polyplex efficacy strongly depends on an optimized combination of core polymers and shell functionalities which can tune efficiency by several log units.

4. NEXT STEPS: MULTIFUNCTIONAL AND SEQUENCE-DEFINED POLYMERS

As reviewed above, sophisticated multifunctional virus-like carriers may be the solutions for overcoming the many different delivery hurdles (Wagner, 2004). This creates a new significant challenge to the macromolecular chemistry: ideally, precise polymers with low or no polydispersity and defined conjugation sites for orthogonal conjugation chemistries should be available. In reality, delivery domains often have been chemically conjugated with polymers in a rather random fashion. Polydispersity and lack of defined numbers of orthogonal conjugation sites make the synthesis of conjugates of more than three components almost impossible with respect to reproducible production.

Nature has demonstrated how to solve the multifunctionality problem with macromolecular structures of high precision. The various delivery microdomains of natural viruses have been generated as defined protein and lipoprotein sequences made from precise translation process from sequences stored as genetic sequence formation. The delivery domains have been optimized by biological evolutions of such sequences. Macromolecular chemistry has made tremendous progress over the last decades; based on automated solid-phase-assisted synthesis nowadays it is routine to synthesize peptide and oligonucleotide sequences, and even the syntheses of whole proteins (Wang et al., 2013) and a whole bacterial DNA genome (Gibson et al., 2010) has been achieved. Therefore, could a natural sequence-based production and evolution process of viral macromolecule design be applied to artificial chemical carrier systems? The requirements are as follows: (1) identification of novel building blocks which serve as microdomains particularly useful in specific delivery processes; these can be nature-derived units such as amino acids or lipids or completely artificial units; (2) assembly in various defined macromolecular sequences; evaluation in delivery and other relevant bioassays; (3) analysis of structure–activity relations, followed by modification or shuffling of encouraging macromolecules into novel sequences; (4) several rounds of evolutionary selection. Some elements of such a "chemical evolution" process have already been implemented by various researchers.

Nice sequence-defined structure–activity relations were made for peptide-based transfection agents (Chen, Kim, Steenblock, Liu, & Rice, 2006;

Chen, Zhang, Stass, & Mixson, 2001; Leng & Mixson, 2005; Leng et al., 2005; McKenzie et al., 2000; Stevenson et al., 2008; Wadhwa, Knoell, Young, & Rice, 1995; Wang et al., 2007), delivering defined linear or branched structures including nucleic acid-binding amino acids such as lysines and arginines, polyplex-stabilizing cysteines, or endosomal-buffering histidines for DNA and siRNA delivery. Analogous to classical peptide synthesis, Hartmann, Börner, and colleagues have applied solid-phase-assisted macromolecule synthesis for assembly of protected diamines and diacids into poly (amido amines) (Figure 8.6(A)) used for DNA polyplex formation (Hartmann & Börner, 2009; Hartmann, Hafele, Peschka-Suss, Antonietti, & Borner, 2008;

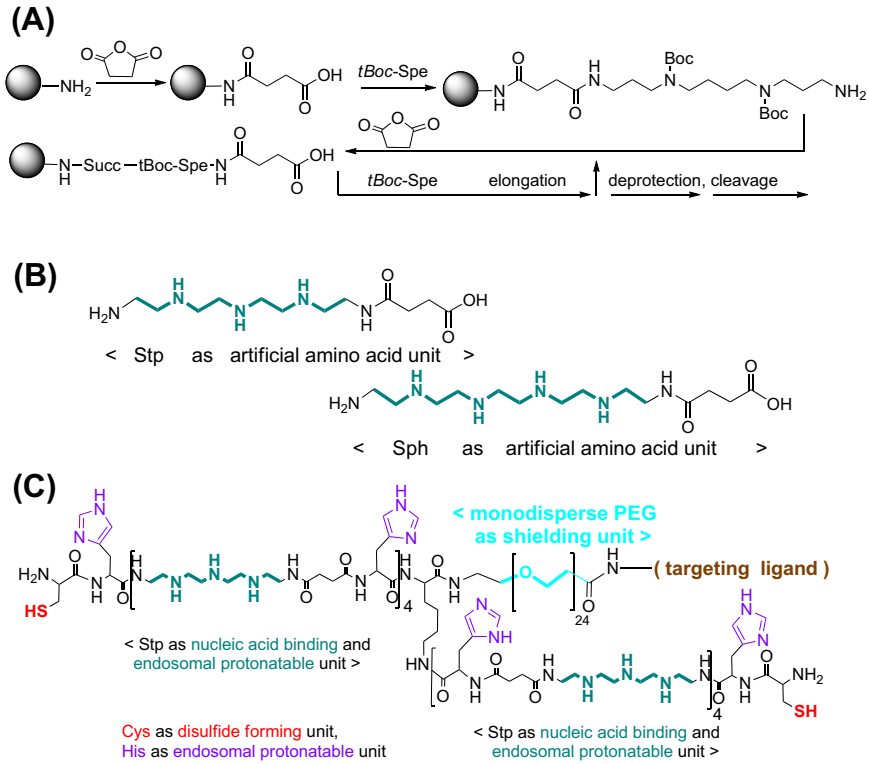

Figure 8.6 ***Sequence-defined polymers.*** (A) Sequential assembly of polymers on solid-phase by alternative coupling of dicarboxylic acids and diamines (L. Hartmann et al., 2006). (B) Stp and Sph as artificial oligoamino acid units (Schaffert, Badgujar, et al., 2011) are applied in solid-phase synthesis in proper tBoc and Fmoc protected form (not shown). (C) Example of a ligand-containing, sequence-defined targeting oligomer containing PEG for shielding, Stp units and histidines for endosomal buffering, and terminal cysteines for oxidative bioreversible polyplex stabilization (Lachelt, Kos, et al., 2014).

Hartmann, Häfele, Peschka-Süss, Antonietti, & Börner, 2007; Hartmann, Krause, Antonietti, & Borner, 2006). Extending this strategy, artificial oligoamino acids (Figure 8.6(B)) have been generated in tBoc, Fmoc-protected form which contains the very effective diaminoethane repeat of the PEI structure as chemical microdomain (Schaffert, Badgujar, & Wagner, 2011). These novel building blocks can be assembled with classical peptide synthesis methodology into various sequences and topologies (Schaffert, Troiber, et al., 2011; Schaffert, Troiber, & Wagner, 2012). The chemically precise design allows addressing simple questions on structure–activity relationships. For example, linear sequences of the building block Stp (succinyl tetraethylene pentamine containing three protonatable nitrogens per unit) were generated to investigate the effect of increasing molecular weight of linear oligo(ethanamino) amides (Scholz et al., 2014). Beyond a critical length of more than 10 Stp units, efficient DNA polyplex formation as well as gene transfer occurred. At an optimum length of 30 Stp units, representing 90 protonatable nitrogens, a sixfold higher transfection efficiency was observed compared with standard linear PEI 22 kDa, which contains approximately 500 (±200) protonatable nitrogens. Importantly, cytotoxicity was 10-fold lower for the oligomers with 30 Stp units than for linear PEI.

A series of lessons previously learned with conventional polymeric carriers could easily be utilized for design by the solid-phase-assisted technology, resulting in more sophisticated multifunctional but still precise sequence-defined DNA and siRNA carriers. For example, targeting ligands in the form of peptide sequences (Lachelt, Kos, et al., 2014; Martin et al., 2012) or smaller molecules such as folic acid (Dohmen et al., 2012) or methotrexate (Lachelt, Wittmann, et al., 2014) attached via precise PEG domains for shielding could be assembled with defined oligoamine carrier topologies such as two-arm or four-arm structures (Figure 8.6(C)). Targeting and shielding domains could be included within the same solid-phase synthesis or, alternatively, be attached to core oligomers by site-specific native chemical peptide ligation reaction (Zhang et al., 2014).

Based on previous studies (Frohlich et al., 2012; Read et al., 2003), oligomers were provided with terminal cysteines for bioreversible polyplex stabilization (Dohmen et al., 2012; Fröhlich et al., 2012; Salcher et al., 2012). Tyrosine residues, which had been found to convert PEI into effective siRNA delivery agents (Creusat et al., 2010), were also improving novel sequence-defined oligomers (Troiber et al., 2013). Also, incorporation of histidines which enhanced endosomal buffering and transfection of various polymers (Bertrand et al., 2011; Gomez et al., 2013; Goncalves et al., 2002; Midoux & Monsigny, 1999)

was found to optimize endosomal escape of novel oligomers with or without targeting and shielding domains (Lachelt, Kos, et al., 2014). In the same report, fine-tuning of the proton sponge characteristics of oligoamine microdomains was possible by providing distinct repeat numbers of the diaminoethane motif. Even numbers of protonatable nitrogens within a microdomain resulted in preferential buffering at endosomal pH 5–6, whereas odd numbers of protonatable nitrogens displayed buffer capacity around neutral pH 7. These observations were well consistent with reports from Kataoka and colleague (Miyata et al., 2012; Uchida et al., 2011) who had designed biodegradable polycations based on side chain amidation of polyaspartic acid with a series of different defined small oligoethylenimines (see also chapter by Takemoto, Miyata, Nishiyama, & Kataoka, 2014).

The ability of synthesizing precise, sequence-defined macromolecular carriers is one important requirement for chemical evolution of polyplexes. Selecting and identifying interesting candidate carriers and subsequent evolution into optimum polyplexes present a more formidable challenge. High-throughput semiautomated screening of libraries of various novel polymers, see for example, the work by Akinc, Anderson, Langer, and colleagues (Akinc et al., 2008; Anderson et al., 2003; Lynn, Anderson, Putnam, & Langer, 2001), is one of the strategies for selection. In addition, by new mixing technologies, novel combinatorial polyplexes can be generated and screened with higher effectiveness. An impressive report was published by Tseng and collaborators using a digital microreactor microfluidic assembly system. Within less than 3 h testing of various polymers in various ratios, they generated a library of more than 600 pDNA/polymer nanoparticles (Wang et al., 2010). Also refined nanoparticle assemblies such as layer-by-layer (Blacklock, Vetter, Lankenau, Oupicky, & Mohwald, 2010) have high potential in further optimization of "synthetic viruses."

Despite the very promising continuous development in the field, great challenges still persist in chemical evolution of polyplexes; in particular, the commonly used in vitro screening methods are not very predictable on the in vivo performance in a whole organism. Therefore identification of selection criteria, which are most relevant for in vivo efficacy and design of corresponding robust screening assays, has critical importance. A recent nice example in this direction was reported by Whitehead et al., establishing four necessary structural and pKa criteria for the prediction of in vivo performance of siRNA lipidoid nanoparticles (Whitehead et al., 2014).

5. PERSPECTIVES

The first five decades of polyplexes were associated with a tremendous improvement in polymer chemistry, nucleic acid biology, and molecular biology, which also triggered a better understanding of transfection processes and their continuous optimization and first therapeutic human clinical trials (Anwer, Barnes, Fewell, Lewis, & Alvarez, 2010; Davis et al., 2010; Fewell et al., 2009; Gofrit et al., 2014; Konstan et al., 2004; Lisziewicz et al., 2012; Ohana et al., 2004; Rodriguez et al., 2013; Schreiber et al., 1999; Sidi et al., 2008). In contrast to recent successful application of viral vectors for gene therapy, which presents a real medical breakthrough, medical translation of polymer-based systems is still moderate. Among other issues, refined processes for chemical evolution of polyplexes would be critically required. It would be interesting to know if the next five decades will solve these issues and provide significant polymer-based nucleic acid medicines.

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CHAPTER NINE

Recent Developments in Nucleic Acid Delivery with Polyethylenimines

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Contents

Abstract

Polyethylenimines (PEIs) have proven to be highly efficient and versatile agents for nucleic acid delivery in vitro and in vivo. Despite the low biodegradability of these polymers, they have been used in several clinical trials and the results suggest that the nucleic acid/PEI complexes have a good safety profile. The high transfection efficiency of PEIs probably relies on the fact that these polymers possess a stock of amines that

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can undergo protonation during the acidification of endosomes. This buffering capacity likely enhances endosomal escape of the polyplexes through the "proton sponge" effect. PEIs have also attracted great interest because the presence of many amino groups allow for easy chemical modifications or conjugation of targeting moieties and hydrophilic polymers. In the present chapter, we summarize and discuss the mechanism of PEI-mediated transfection, as well as the recent developments in PEI-mediated DNA, antisense oligonucleotide, and siRNA delivery.

1. INTRODUCTION

Gene delivery has shown potential in a wide variety of applications, including basic research and therapies for genetic and acquired diseases. However, the success of gene therapy is largely dependent on the development of efficient DNA carriers. One delivery approach consists in exploiting the properties and tropisms of viruses. This is currently the most widely used system, including in clinical trials. All other approaches, collectively termed "nonviral gene delivery systems" try to mimic the efficiency of viral vectors by artificial means. Among those, the most explored strategy consists in using structurally well-defined synthetic compounds, which are most often cationic. Several thousands of synthetic cationic compounds, essentially lipids and polymers, have been developed for the delivery of nucleic acids in the last 25 years. Ethylenimine polymers (polyethylenimines (PEIs)) belong to the most popular transfection agents. Indeed, although the original paper reporting the transfection capacities of PEI was published almost 20 years ago (Boussif et al., 1995), PEI is still considered one of the best vectors to deliver genes and often serves as "gold-standard" for those who develop new DNA carriers. This is exemplified by the 3500 citations of Jean-Paul Behr's article describing gene transfection with PEI in 1995.

Why do PEIs attract such great interest? An obvious reason is that this family of polymers belongs to the most efficient synthetic gene transfer agents developed so far. But there are also several other reasons: (1) PEI is a robust transfection agent, meaning that it transfects many cell lines in a reproducible manner; (2) the protocol for generating the DNA complexes is extremely simple; (3) PEI solution is stable for years even at 4 °C and does not need particular storage conditions—which is not the case for all the vectors, in particular for peptides and for some lipids that are prone to degradation; (4) these cationic polymers are commercially available in a wide range of molecular weights as well as in a linear and branched topology and they are not expensive; and (5) finally, due to the presence of many amino groups, chemical modifications of these polymers or conjugation of targeting groups or PEGylation are relatively easy.

Here, we review and discuss the mechanism of PEI-mediated DNA transfection as well as the recent developments in PEI-mediated DNA, antisense oligonucleotide, and siRNA delivery.

2. MECHANISM OF PEI-MEDIATED DNA TRANSFECTION

Despite various studies aimed at investigating the mechanism of uptake and intracellular trafficking of nonviral vectors, the processes involved in PEI-mediated gene delivery are still not completely clear. For example, the manner how PEI allows for endosomal escape of the nucleic acid remains debated (see next section). An additional complication comes from the fact that not all the PEIs behave in the same way: the most commonly used PEIs for transfection are the linear PEI 22 kDa (L-PEI) and the branched PEI of 25 kDa (B-PEI). In contrast to the B-PEI, the size of L-PEI polyplexes strongly depends on the salt concentration during preparation. In the presence of physiologic salt concentrations, L-PEI polyplexes form large aggregates, while small particles (<100 nm in diameter) are formed in low-salt buffers. This difference in behavior is not anecdotic since L-PEI mediates significantly higher in vivo reporter gene expression after intravenous injection than the branched polymer (Wightman et al., 2001).

However, globally, the mechanism of PEI-mediated gene delivery can be summarized as follows.

PEIs interact efficiently with nucleic acids due to the presence of charged amino groups. Examination of B-PEI 25 kDa/DNA complexes using electron microscopy revealed a homogenous population of essentially toroidal particles with a size range of 40–60 nm (Tang & Szoka, 1997). But, as mentioned above, the size of the polyplexes may vary depending on the PEI and on the presence or absence of salt (Wightman et al., 2001). Also worth mentioning, when using N/P ratios between 6 and 10, which are usually optimal for gene transfer, a large amount of PEI remains in a free form (Clamme, Azoulay, & Mely, 2003). Whilst these free PEI molecules cause toxicity, they also participate to a large extent to the high transfection efficiency of the DNA-bound PEI (Boeckle et al., 2004; Dai, Gjetting, Mattebjerg, Wu, & Andresen, 2011; Yue et al., 2011). As efficient transfection requires the condensed DNA particles to bear a cationic surface, it was proposed that the DNA complexes interact with the cell surface through the binding to anionic proteoglycans (Labat-Moleur et al., 1996; Mislick & Baldeschwieler, 1996). Syndecans—which are, along with glypicans in

mammals, the dominating cell-surface HSPGs—were shown to be involved in PEI/DNA polyplexes binding (Kopatz, Remy, & Behr, 2004). In particular syndecans-1 play an important role in PEI-mediated transfection (Paris, Burlacu, & Durocher, 2008). Several studies investigated the cellular uptake mechanism of the PEI/DNA complexes (Gabrielson & Pack, 2009; Kopatz et al., 2004; Rejman, Bragonzi, & Conese, 2005). Many discrepancies are found in the literature some of which can be explained by the differences of the experimental conditions that were used (cell lines, polyplex formulation…). Concerning this particular step of PEI-mediated transfection, it is highly probable that the uptake pathway mediating successful gene expression depends on the cell line, type of PEI, and polyplex formulation (i.e., size of the complexes) that are used. This was confirmed in a nice study performed by von Gersdorff et al. (2006). These authors found that uptake of small PEI polyplexes proceeds via at least two different pathways, namely the clathrin- and the caveolae/raft-dependent route. Once endocytosed, the nucleic acids have to escape from the endocytotic vesicles before degradation of the DNA occurs. The high transfection efficiency of PEI in vitro has been ascribed to its ability to buffer the pH in the endosomal compartments and this in turn could induce rupture of the endosomal membranes, resulting in the release of PEI/DNA complexes into the cytosol. This has been called the "proton sponge effect." This part of the process of PEI-mediated transfection will be discussed in more detail in the next section. Once in the cytosol, DNA has to be protected against nuclease-mediated degradation (Lechardeur et al., 1999) and this was shown to be the case when complexed with PEI (Pollard et al., 2001). But remains the question as to whether PEI is complexed to DNA once in the cytosol. Iida and colleagues showed that addition of a cytosolic fraction to B-PEI 25 kDa/DNA complexes did not result in a release of DNA at N/P of 6, or only weakly at N/P 2 (Iida, Mori, Katayama, & Niidome, 2007). Itaka et al. used FRET technology to address this point. They observed DNA decondensation in the cytoplasm when the plasmid was complexed with L-PEI whilst B-PEI remained bound to DNA (Itaka et al., 2004). Then, DNA needs to be transported to the nucleus. There is very few information about the cytoplasmic transport of DNA polyplexes. However, microinjection experiments clearly demonstrated that the cytoplasm imposes a diffusional barrier to nucleocytoplasmic transport of DNA. Indeed, nucleic acids >2 kb were found to be virtually immobile (Lechardeur & Lukacs, 2006; Lukacs et al., 2000). It has been suggested that molecular crowding and collisional interactions are probably responsible for the slowed DNA diffusion. The next

step, nuclear translocation of plasmid DNA, requires either the disassembly of the nuclear envelope during cell division or active nuclear transport via the nuclear pore complex. Alternatively, results of a recent study suggest that PEI is able to permeabilize the nuclear membrane (Grandinetti, Smith, & Reineke, 2012). Whether this nuclear envelope permeability allows entry of the polyplexes into the nucleus, or is the result of apoptosis initiation, remains, however, to be shown. Microinjection experiments showed that less than 1/1000–1500 naked plasmid DNA copies injected in the cytoplasm are effectively trafficked to the nucleus (Lechardeur & Lukacs, 2006; Pollard et al., 1998). Interestingly however, Pollard and co-workers found that 1/100 DNA/B-PEI 25 kDa complexes were trafficked to the nucleus. These results suggest that the complexation of plasmids with PEI either protects the DNA against degradation or enhances its nuclear uptake. The capacity of PEIs to deliver plasmids into the nucleus has also been analyzed by Brunner et al. by transfecting HeLa cells at different stages of the cell cycle (Brunner, Furtbauer, Sauer, Kursa, & Wagner, 2002). For cells transfected with the linear PEI of 22 kDa, the difference between pure G1 and late S cells was less than sixfold after 8 h and only 11-fold after 1 day while the difference in luciferase expression between cells transfected in early and late S was more than 300-fold after 8 h with Lipofectamine. Surprisingly, the results with the B-PEI of 25 kDa were similar to those obtained with the cationic lipid. These results suggest that at least linear PEIs could favor nuclear transport of DNA.

Finally, it has been investigated whether the final step in the gene delivery process, namely gene transcription, can be inhibited if the plasmids remain complexed with PEI once in the nucleus. Results of in vitro transcription assays showed that the condensed DNA retains a transcriptional competence within the PEI complex (Bieber, Meissner, Kostin, Niemann, & Elsasser, 2002; Honore et al., 2005). However, increasing the N/P ratio seems to be detrimental to the transcriptional activity (Maury et al., 2014).

To conclude with the transfection process of PEIs, let us consider the efficiency of some of the key steps: in general, transfection with PEI is often considered to be "efficient." This relies on the fact that cell lines that are commonly used in research laboratories such as HEK293, CHO-K1, B16-F10 can be transfected with an efficiency that ranges between 50% and 80%. But are these percentages sufficient to conclude that the process is efficient? Standard transfection conditions use 10^5 to 10^6 copies of plasmid/cell. It has been shown that 5–20% of the nucleic acids are taken up by the cells (Cohen, van der Aa, Macaraeg, Lee, & Szoka, 2009; Kichler,

Leborgne, Coeytaux, & Danos, 2001). Unfortunately, quantitative determination of DNA copy number escaping the endosomal compartment is not feasible. However, fluorescent and electron microscopic observations, as well as cell fractionation data indicate, that PEI–DNA complexes accumulate in the lysosomal compartment. This suggests that only a small fraction of the complexes reach the cytosol (Bieber et al., 2002). Quantification of plasmid DNA copies in the nucleus after PEI transfection has been achieved recently (Cohen et al., 2009). The authors determined that transfected cells contained 75 to 50,000 plasmids/nucleus, depending on both, the initial dose of DNA and the cell line. This means that, in the best case (i.e., using cell lines that are permissive to PEI-mediated transfection), 1–5% of the applied dose of DNA is delivered in the nucleus. This probably explains why PEI/DNA complexes can hardly be detected in the cell nuclei when using confocal microscopy (Bieber et al., 2002; Maury et al., 2014). A comparison with what can be achieved with recombinant viral vectors will give us an idea of the efficiency of the transfection process: the transduction efficiency of a lentiviral vector encoding the eGFP reporter gene has been evaluated in 11 human lung cancer lines at increasing Multiplicity of Infections (MOIs; ratio of the number of infectious virus particles to the number of cells). At a MOI of 5, lentiviral transduction resulted in 85–100% eGFP positive cells for 5 out of the 11 cell lines (Chen, Akerstrom, Baus, Lan, & Breslin, 2013). Similar experiments conducted on five tumor cell lines with a recombinant adenovirus showed that around 90% of transduction efficiency was obtained using an MOI of 100 (Teschendorf, Warrington, Shi, Siemann, & Muzyczka, 2006). These results underscore the low efficiency of the transfection process using synthetic vectors as compared to viral transduction. This shows also that significant improvements remain feasible.

3. PROTON SPONGE: MYTH OR REALITY?

A possible explanation for the relatively high gene transfer efficiency of PEI is that the buffering of the endosomes through PEI leads to a massive proton accumulation followed by passive chloride influx. These events should cause osmotic swelling and subsequent endosome disruption (phenomenon called "proton sponge" effect by J-P. Behr (Boussif et al., 1995)), thus permitting the escape of endocytosed materials. In 1997, Behr added another effect that could act in synergy for endosomal release namely expansion of PEI's polymeric network by internal charge repulsion (Figure 9.1; Behr, 1997). The validity of the PEI-mediated proton sponge activity has

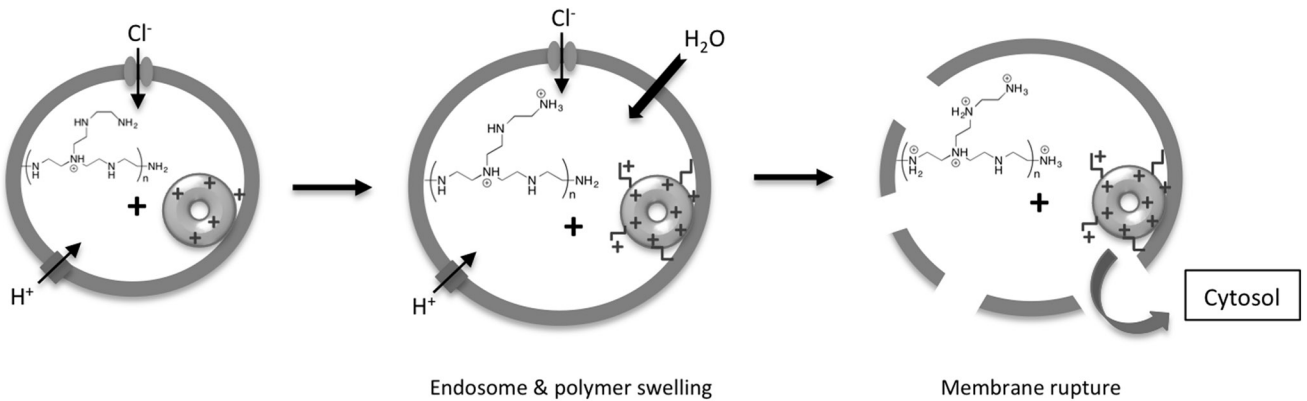

Figure 9.1 The proton sponge effect. Acidification of the endosomes occurs with entry of protons. The accumulation of protons brought in by the endosomal ATPase is coupled to an influx of chloride anions. In the presence of PEI/DNA and free PEI there will be a large increase in the ionic concentration within the endosome resulting in osmotic swelling of the endosome. Due to internal charge repulsion, the polymer will also expand ("umbrella" effect). Altogether, this will lead to rupture of the endosomal membrane and release of the polyplexes into the cytosol.

however been repeatedly questioned. In this section, we will summarize the arguments in favor of this hypothesis and briefly discuss some of the arguments that are put forward to question the hypothesis.

3.1 Is PEI a Proton Sponge?

This hypothesis is based on the chemical structure of PEIs: they differ from other polymers, such as polylysine, in that only a fraction of the amino groups are protonated at physiological pH. And according to their pKa profile, PEIs exhibit considerable buffer capacity over almost the entire pH range (Suh, Paik, & Hwang, 1994; Tang & Szoka, 1997). Although discrepancies still exist concerning the percentage of amines that are protonated at physiological pH (probably around 50%) (Ziebarth & Wang, 2010), it is admitted that PEI does indeed possess the capacity to capture protons entering the endosomes during the acidification process. This property is involved in the transfection process since quaternization of the amines reduces by 50-fold the efficiency of PEI (Akinc, Thomas, Klibanov, & Langer, 2005).

However, a buffering capacity is not sufficient to make a molecule a good carrier for gene delivery. Indeed, various vectors including polymers (Funhoff et al., 2004), intact polyamidoamine dendrimers (Tang, Redemann, & Szoka, 1996), histidine rich peptides (Kichler, Leborgne, Marz, Danos, & Bechinger, 2003), oligo(ethanamino)amides (Lachelt et al., 2014), that were designed for possessing buffering capacities at low pH revealed poorly efficient for DNA transfection. One difference with PEI may be that this polymer exhibits a buffering capacity over a broad pH range, which is not the case for all these vectors. Also, one has to keep in mind that for the proton sponge effect to occur, several conditions have to be combined (see below): a sufficiently high polymer concentration in the endosomes, a capacity to interact with membranes, and probably a significant flexibility (capacity for the polymer to swell). For example, the reason why short chain PEIs are much less effective than long ones although both possess the same buffering capacities at a given N/P lies probably in the fact that short chain B-PEI/DNA complexes are internalized much less efficiently than polyplexes prepared with the 25 kDa B-PEI (Kang, Kang, & Bae, 2011).

3.2 Role of Acidification in the Transfection Efficiency

To study more precisely the role of acidification in the transfection process of PEIs, inhibitors of the vacuolar type H^+-ATPases such as bafilomycin A1 have been used. The results showed that in the presence of the drug, the transfection efficiency is reduced by 7- to 200-fold, depending on the

cell line (Akinc et al., 2005; Kichler et al., 2001; Yue et al., 2011). These data clearly indicate that the acidification process is involved in the PEI-mediated transfection mechanism. However, these results also show that the extent to which the proton sponge is implicated in the escape from the endosomes is cell-type dependent. This cell-type dependency may be explained by differences of the cell uptake pathway and/or cell-specific endosomal characteristics such as endosomal pH or membrane composition. Nevertheless this also suggests that PEIs probably possess, at least under certain conditions, other means to avoid entrapment in endosomes.

3.3 Vesicle Swelling and Rupture of the Membrane

In 2002 Verkman and colleagues provided experimental evidence that endosomal acidification is accompanied by Cl^- entry and endosome swelling (Sonawane, Thiagarajah, & Verkman, 2002). Using the same techniques, Verkman et al. designed experiments to test the validity of the proton sponge hypothesis. They found that acidification of the endosomes containing PEI/DNA complexes was reduced whereas Cl^- accumulation and swelling was increased (Sonawane, Szoka, & Verkman, 2003). Furthermore they found that endosomes containing PEI/DNA—but not polylysine/DNA complexes—had an increased buffer capacity and showed an increased osmotic fragility. Interestingly, endosomes containing polylysine/DNA adopted similar characteristics than those containing PEI after addition of the weak base chloroquine (the two pK values of chloroquine are 10.2 and 8.1 and therefore at physiological pH, only 82% is biprotonated) to the culture medium. Finally, Verkman et al. followed individual endosomes containing fluorescently labeled polyplexes and they observed that some of the vesicles disappeared probably as a consequence of lysis. Several of these findings were confirmed by other groups. For example, swelling and bursting of a small percentage of endosomes/lysosomes containing PEI/ribozyme complexes has also been reported by Merdan et al. (Merdan, Kunath, Fischer, Kopecek, & Kissel, 2002). Another study based on electron microscopical analysis of endosomes/lysosomes containing PEI/DNA polyplexes revealed membrane damages of variable size (Bieber et al., 2002).

One interesting question is what is the range of concentration required to induce bursting of endosomes/lysosomes since this parameter directly influences the buffering capacity? Won and co-workers calculated that the osmotic pressure induced by PEI during the acidification of the endosome is theoretically insufficient to cause endosome disruption (Won, Sharma, & Konieczny, 2009). The authors estimated that ethylenimine

(EI) concentration in endosomes lies around 70 mM while concentrations above 150 mM are necessary to build up an osmotic pressure strong enough for breaking the lipid membrane bilayer. Conversely, Benjaminsen et al. determined experimentally that in <1% of lysosomes the concentration of EI reaches >300 mM (Benjaminsen, Mattebjerg, Henriksen, Moghimi, & Andresen, 2013). This latter value is in agreement with the theoretical calculations from Pinel et al. (2014).

3.4 pH Environment of PEI-Based Polyplexes

The team of Langer measured by flow cytometry, the average pH environment of PEI once in the cell and found that 2 h after incubation with the polyplexes it was around 6 with PEI/DNA while for polylysine/DNA the average pH was 4.5 (Akinc & Langer, 2002; Akinc et al., 2005). More recently, Benjaminsen and colleagues measured by using a newly developed nanoparticle-based pH sensor the lysosomal pH after addition of PEI and they observed no changes in pH (Benjaminsen et al., 2013). Although these results are not in agreement with the ones of Akinc et al. (Akinc & Langer, 2002; Akinc et al., 2005), it does not exclude that PEIs act through the proton sponge effect. Indeed, as mentioned by the authors of the study, there are several possible explanations: (1) the complexes escape the endosomal pathway before they reach the lysosomes, (2) the complexes escape by the proton sponge mechanism but without changing the pH of the lysosomes. This hypothesis seems reasonable because the V-ATPase will continuously pump protons into the vesicles—which will inexorably lead to acidification once PEI is fully protonated.

3.5 Polymer Swelling and Membrane Interactions

The effect of protonation on the conformation of linear PEI has been studied and the results show that the PEI chain adopts a conformation that increases the distance between the positive charges—in order to reduce electrostatic repulsion (Choudhury & Roy, 2013; Ziebarth & Wang, 2010). In other words, the PEI chain elongates when the protonation level increases. This is in good agreement with the results obtained by Tang et al. with fractured PAMAM dendrimers and PEI. Indeed, they observed for both polymers an increase of the volume when decreasing the pH while it was not the case for intact dendrimers which are much less efficient for DNA transfection than degraded PAMAMs (Tang, Redemann, & Szoka, 1996). Based on these results, Szoka proposed an extension of the proton sponge effect with the umbrella hypothesis which describes the ability of polymers

to expand volumetrically when protonated at acidic pH (see Figure 9.1; Nguyen & Szoka, 2012; Tang et al., 1996). Recently, results of a computational simulation of the behavior of linear PEI in different protonation states with DOPC molecules describe how protonation and elongation of L-PEI could intervene in the umbrella effect (Choudhury, Kumar, & Roy, 2013). Indeed, the results indicate that the protonated PEI chain interacts with the lipid bilayer and this in turn leads to formation of hydrophilic pores. Interestingly, it has been reported that free PEI molecules as well as PEI/DNA complexes remain bound to the inner membrane face of the endosomes (Clamme, Krishnamoorthy, & Mely, 2003). These interactions may lead to local lipid bilayer destabilization. This hypothesis is plausible since it has been shown that PEI is able to permeabilize Gram negative bacterial outer membrane (Helander, Alakomi, Latva-Kala, & Koski, 1997) as well as the cytoplasmic face of lysosomal membranes (Klemm, Young, & Lloyd, 1998).

Altogether, these results support the fact that PEIs act, at least partially, through the proton sponge effect, and probably also polymer swelling, to induce endosomal release of the cargo. A theoretical investigation of the proton sponge hypothesis confirms the feasibility of this mechanism—but only if a certain amount of free PEI is present in the vesicles (Yang & May, 2008). Also in agreement with J-P Behr's hypothesis is the fact that the efficiency of different delivery systems could be improved by the addition of chemical groups having a pKa below 6.5. For example, the transfection efficiency of a polylysine partially substituted with histidyl residues that become cationic upon protonation of the imidazole groups at pH below 6 is strongly enhanced (Midoux & Monsigny, 1999). What seems also clear is that most of the internalized PEI/DNA complexes reach the lysosomes and that only a small fraction escapes from the endosomes/lysosomes. This is probably due to the fact that several conditions (concentration of PEI, ratio of free- and DNA-bound PEI, size/shape/composition/pH of the endosome) have to be met in order for the proton sponge and umbrella effects to enable permeabilization of the membranes.

4. PEI FOR GENE DELIVERY

4.1 Recent Developments

As mentioned previously, a large variety of PEI derivatives have been synthesized and evaluated for their capacity to deliver genes into cells. It is beyond the scope of this chapter to discuss all these modifications. We will focus on four approaches that we believe to be important.

- One important focus in the last years was to reduce the toxicity of the PEIs (Moghimi et al., 2005). Low MW (LMW) PEIs are much less efficient than PEIs of >20 kDa while being less cytotoxic. A strategy that has been explored by several laboratories consisted in the conversion of LMW PEIs into high MW PEI through cross-linking with biodegradable linkages such as disulfide bridges. The idea behind is to enable the cell to transform a high MW PEI into low MW PEI fragments that could be eliminated more easily and this in turn should allow reducing the cytotoxicity of the polymer. Different cross-linking strategies have been evaluated using various linear and branched LMW PEIs (for review see Son, Namgung, Kim, Singha, & Kim, 2012). The results are promising since several compounds could be identified that have a lower cytotoxicity while having an overall gene transfection efficiency that is globally comparable to that of the B-PEI 25 kDa or the L-PEI 22 kDa (Breunig, Lungwitz, Liebl, & Goepferich, 2007; Deng et al., 2009; Gosselin, Guo, & Lee, 2001; Kang et al., 2011; Lee et al., 2007; Peng, Hu, Cheng, Zhong, & Zhuo, 2009).
- Another aspect that has been intensively studied in the last years is the shielding of the surface charge of the particles by hydrophilic polymers—in particular polyethylene glycols (PEGs). The objective of this hydrophilic coating is to diminish the interactions of the particles with blood components and to reduce the uptake by macrophages and this in turn should result in an increased blood circulation time. Such modifications belong to what has been called the "stealth technology." Results have shown that PEGylation of PEIs leads to an increased solubility of the complexes as well as to a reduction in the surface charge of the polyplexes (Kichler, Chillon, Leborgne, Danos, & Frisch, 2002; Nguyen et al., 2000; Petersen et al., 2002). These modifications of the properties of PEI allow the injection of highly concentrated formulations and the reduction of deleterious interactions of the particles with blood components. However, PEGylation also reduces the DNA-binding capacity of the polymer and it sterically hinders interactions of the polyplexes with the target cells. This results in reduced in vitro transfection efficiency. Despite this reduced capacity of interaction with cells, Zhou and co-workers obtained a significant and selective transfection of tumors in mice. The formulation developed by these authors consisted in a core of HMW L-PEI complexed with DNA and surrounded by a shell of PEG-modified LMW L-PEI (Yang et al., 2013). The preferential transfection of tumors may be due to EPR-like effects (enhanced permeability and retention).

- Many efforts that have been made in view of in vivo applications consisted in combining the stealth technology with the use of ligands that allow specific cell targeting. Various ligands have been used including galactose (Sagara & Kim, 2002), transferrin (Kursa et al., 2003), folate (Benns, Mahato, & Kim, 2002), and epidermal growth factor (Blessing, Kursa, Holzhauser, Kircheis, & Wagner, 2001). The team of Ernst Wagner made a major contribution to this field of research by studying the influence of different parameters as for example pre-and post-PEGylation (Blessing et al., 2001; Ogris et al., 2003). In vivo evaluation of PEI-PEG-ligand based formulations support the idea that these modifications can improve the biodistribution. For example repeated intravenous administration of the targeting conjugate L-PEI-PEG2kDa-EGF associated with the cytotoxic double-stranded RNA polyinosinic:polycytidylic acid (poly(I:C)) strongly retarded growth of A431 human tumor xenograft in mice (Schaffert et al., 2011).
- Finally, a field that attracts great interest today is the development of multifunctional nanocarriers that combine both therapeutic and diagnostic capabilities. Recently, it has been reported that carbon dots with PEI-passivation are efficient nanocarriers for siRNA and DNA while possessing properties allowing bioimaging (Liu et al., 2012; Wang et al., 2014). These latter findings will for sure rapidly lead to the development of new PEI-based nanomaterials.

4.2 PEI in Clinical Trials

Among the thousands of synthetic vectors that have been designed, only few of them—including PEI—have been used as delivery systems in clinical trials. Here, we will briefly list the clinical trials that have been performed/are underway using PEI or derivatives thereof (see also http://www.polyplus-transfection.com/therapeutics/clinical-pipeline/ and ClinicalTrials.gov).

1. Bladder cancer: H19 is a gene expressed in 85% of bladder tumors but suppressed in the adult healthy bladder. BC-819 (also called DTA-H19) is a DNA plasmid that carries the gene for diphtheria toxin-A under regulation of the H19 promoter sequence. A phase I/IIa and a phase IIb have been performed that consisted in intravesical administration into the bladder of BC-819 complexed with linear PEI (Gofrit et al., 2013; Sidi et al., 2008). The results of these two trials indicate that L-PEI/BC-819 has a good safety profile with a low rate of adverse effects (Gofrit et al., 2013).

2. Pancreatic adenocarcinoma: a pilot study for the treatment of locally advanced pancreatic adenocarcinoma with intratumoral injection of linear PEI/DNA complexes with antitumoral effects and chemosensitizing activity for Gemcitabine has been recently completed. The protocol consisted in the intratumoral administration of increasing doses of a plasmid DNA complexed to L-PEI that encodes two genes (somatostatin receptor subtype 2 and deoxycytidine kinase: uridylmonophosphate kinase). Both transgenes are supposed to induce an antitumor bystander effect and render gemcitabine treatment more efficient.
3. B-cell malignancies: the purpose of the study is to determine how well SNS01-T is tolerated by relapsed or refractory multiple myeloma, B-cell lymphoma, or plasma cell leukemia patients when given by intravenous infusion at various doses. SNS01-T, is an L-PEI-based nanoparticle comprised of both an RNAi-resistant DNA plasmid expressing non-hypusinable $eIF5A_{K50R}$ and an eIF5A siRNA to reduce expression of endogenous hypusinated eIF5A (Francis et al., 2014).
4. Acute myocardial infarction: objective of this phase IIb study: enhance the function of autologous progenitor cells by overexpressing endothelial nitric oxide synthase. Circulating mononuclear cells are transfected ex vivo with a human eNOS-pVAX plasmid complexed with L-PEI (Taljaard et al., 2010). The transfected progenitor cells are then re-infused into patients by coronary injection into the infarct-related artery.
5. Therapeutic HIV vaccine: Several clinical trials (Phase I, phase II) have been performed/are underway with DermaVir which is a DNA medicine that is topically administered (Lisziewicz et al., 2012; Rodriguez et al., 2013). DermaVir is a formulation containing a plasmid DNA encoding for 15 HIV antigens that is complexed with a linear 22 kDa PEI modified with mannose residues. The objective of the immunization with DermaVir is to improve viral antigen presentation via dendritic cells/Langerhans cells. Clinical trials allowed the conclusion that DermaVir is safe and results suggest that this vaccine boosts T cell responses against HIV antigens expressed from the DNA.
6. Recurrent ovarian cancer: a PEG-PEI-cholesterol (PPC) derivative (Fewell et al., 2005) complexed with an interleukin-12 expressing plasmid has been evaluated in a phase I trial with patients with recurrent ovarian cancer (Anwer et al., 2013). The DNA complexes were injected intraperitoneally. Another phase I has started which aims to study the side effects and the best dose of giving EGEN-001 together with PEGylated liposomal doxorubicin.

These first clinical results give reason for optimism. Importantly, the gene medicine containing PEIs or derivatives seem to have a reasonably good safety profile with adverse effects that remain compatible with human use.

5. PEI DERIVATIVES FOR THE DELIVERY OF EXON SKIPPING OLIGONUCLEOTIDES

Many different human diseases are caused by errors in pre-mRNA splicing. Antisense oligonucleotide therapies show particular promise in this area. Studies have been initiated to restore the reading frame transcripts in patients suffering from Duchenne muscular dystrophy. Interesting results were obtained in the dystrophic mouse model mdx following intramuscular or IV injection of 2′O-Methyl phosphorothioate oligonucleotides (Heemskerk et al., 2009). Nevertheless, only a small proportion of the exon-skipping oligonucleotides (ESOs) are delivered to the target cells. Thus, increasing the delivery efficiency is important for the success of this approach. *In vitro* experiments using HeLa and C2C12 cells indicated that B-PEI 25 kDa is rather inefficient for the delivery of ESO (Wang, Wu, et al., 2014; Zaghloul, Viola, Zuber, Smith, & Lundin, 2010). Intramuscular injection of B-PEI 25 kDa/ESO in mdx mice resulted also in a low exon skipping efficiency (Wang, Wu, et al., 2014). These results suggest that PEI 25 kDa is a better vector for plasmid DNA than for short oligonucleotides. Lutz and colleagues evaluated the capacity of various PEI-PEG copolymers to deliver ESOs in vivo, after intramuscular injection of the complexes into mdx mice (Sirsi et al., 2008; Williams, Sirsi, Latta, & Lutz, 2006). They found that PEGylated low molecular weight PEI (2 kDa) allowed for a significant increase in the number of dystrophin positive fibers (consequence of the exon skipping) as compared to naked antisense oligonucleotides. More recently, similar experiments have been conducted using various copolymers of Pluronic-low molecular weight PEI (Wang, Wu, et al., 2014). The authors identified two copolymers that were particularly effective for exon skipping in mdx mice, namely Pluronic L44-PEI 800 Da and Pluronic L35-PEI 1200 Da. Taken together, these results demonstrate that low molecular weight PEIs modified by PEGs or Pluronics are significantly more efficient than the B-PEI 25 kDa for the delivery of ESOs in muscles fibers after intramuscular injection.

Another strategy that has been used to improve the efficiency of PEIs for vectorization of ESOs consists in modifying the PEI with hydrophobic groups such as tyrosine (PEI-Y). These modifications transform the soluble

PEI into a self-aggregating molecule and this in turn increases the cohesion of the PEI/oligonucleotide complexes (Creusat & Zuber, 2008). The efficiency of PEI-Y is indeed higher than that of PEI in vitro (Zaghloul et al., 2010).

6. PEIS FOR SIRNA DELIVERY

6.1 PEI without Modifications

Highly efficient DNA carriers are not necessarily suited for siRNA delivery. Indeed, while the B-PEI of 25 kDa and the linear PEI of 22 kDa are recognized as belonging to the most efficient plasmid DNA transfection agents, these polymers are rather poorly efficient for the delivery of dsRNA duplexes (Grayson, Doody, & Putnam, 2006). The results of the siRNA transfection studies indicate that only the 25 kDa B-PEI complexed with siRNA was capable of mediating specific RNA interference at high concentrations (Grayson et al., 2006). Successful in vitro siRNA delivery was observed at 10-fold higher siRNA concentrations than needed in combination with lipoplexes for instance (Grayson et al., 2006). While siRNA duplexes and genes share a similar anionic charge density, the lower number of anionic charges in a siRNA duplex (21 phosphate groups per single strand) in comparison to plasmid DNA reduces the electrostatic cohesion of the PEI with siRNA, due to reduced cooperative binding, which could explain their low delivery. The siRNA molecule also shares a less flexible, rod-like structure reducing its interaction with the polymer backbone. Back-folding of the linear PEI chain is thermodynamically unpropitious due to the loss of entropy in the flexible polymer chain. Consequently, polyanionic proteoglycans present outside the cells and on the cell surfaces may effectively displace PEI from the complexes, resulting in release of siRNA in the extracellular medium (Bolcato-Bellemin, Bonnet, Creusat, Erbacher, & Behr, 2007).

Despite these theoretical considerations and its low in vitro efficacy, the linear PEI has been successfully used in a subcutaneous mouse tumor model. Intraperitoneal administration of complexed siRNAs targeting a proto-oncogene (HER-2 receptor) resulted in a marked reduction of tumor growth in a subcutaneous xenograft model in mice (Urban-Klein, Werth, Abuharbeid, Czubayko, & Aigner, 2005).

An interesting strategy for enhancing the efficiency of PEIs consists in copying the extended DNA structure and translating it to siRNAs, making them more "gene-like." By introducing self-complementary and overhanging

nucleotides, Jean-Paul Behr's group transformed short siRNA duplexes into longer, plasmid-like, structures called "sticky siRNAs" (ssiRNA) that auto-oligomerize on contact with charged PEI backbone. This innovative concept allowed for significantly increased delivery of siRNAs by L-PEI in vitro (Bolcato-Bellemin et al., 2007). Systemic injection of ssiRNA-PEI complexes in vivo did not lead to any pro-inflammatory cytokine induction and avoided so the interferon response often associated with long double-stranded RNA prone to activate Toll-like receptors (Bonnet, Erbacher, & Bolcato-Bellemin, 2008). Sticky siRNAs complexed with linear PEI have successfully been transferred to in vivo tumor models: ssiRNAs targeting cell cycle regulator proteins (survivin and cyclin B1) have shown antitumoral effect on melanoma subcutaneous xenografts (Kedinger et al., 2013). Similar ssiRNA-PEI complexes were able to inhibit lung tumor metastases in an aggressive mammary adenocarcinoma model (Bonnet et al., 2013). At the same time Park and colleagues developed chemically self-crosslinked and multimerized siRNAs. These double-stranded RNAs were oligomerized via cleavable disulfide linkers and demonstrated enhanced gene inhibition. They produced more stable and compact polyelectrolyte complexes than unmodified RNAs and they showed greatly enhanced gene-silencing efficiencies in vitro and in vivo (Lee, Mok, Jo, Hong, & Park, 2011; Mok, Lee, Park, & Park, 2010).

6.2 Modified PEIs for siRNA Delivery

Different strategies have been evaluated for increasing the capacity of PEIs to deliver siRNAs. We can distinguish four approaches: (1) cross-linking with stable covalent groups, (2) cross-linking with metabolically labile bonds (disulfide linkers, labile ester groups), (3) electrostatic stabilization with a third partner molecule, and (4) introduction of self-assembling hydrophobic groups.

6.2.1 Cross-linking with Stable Covalent Groups

Linear PEI (25 kDa) has been covalently cross-linked with bis-epoxyde linker 1,4-Butanediol Diglycidyl Ether to polyethylenimine (linear PEI, 25 kDa) in order to create a loose mesh of reticulated polymer forming so-called "linear PEI nanoparticles." This tethered material showed 86% inhibition in a co-delivery assay with a plasmid encoding a reporter gene (Goyal et al., 2012; Swami, Kurupati, et al., 2007).

6.2.2 Cross-linking with Metabolically Labile Bonds

Ernst Wagner's group developed cross-linked metabolically labile PEIs named HD-Os (1.6-Hexanediol Diacrylate cross-linked Oligoethyleneimines)

that effectively transfected plasmid DNA in vivo with good biocompatibility (Russ et al., 2010). These molecules were surprisingly poor siRNA delivery vectors. A second step of chemical cross-linking with dithiol containing DSP (dithiobis-succinimidylpropionate) done on top of preformed complexes of siRNA with HD-Os enhanced dramatically their stability and improved their delivery potential. The resulting dithiol linker is readily cleaved in the reductive intracellular environment (rich in free thiol containing molecules like glutathione) releasing the cargo siRNA into the cytoplasm (Frohlich, Edinger, Russ, & Wagner, 2012).

6.2.3 Electrostatic Cross-linking

This concept consists in the stabilization of the polyplexes by electrostatic interactions with a third partner molecule. B-PEI (25 kDa) was converted into nanoparticles through electrostatic interactions with anionic polysaccharides (e.g., alginic acid and hyaluronic acid). Both series of nanoparticles exhibited high siRNA co-delivery with reporter gene with enhanced cell viability as compared to PEI alone (Patnaik, Arif, Pathak, Singh, & Gupta, 2010). Similar results were obtained with PEI and γ-polyglutamic acid nanocomposites (Tripathi et al., 2011). Electrostatic stabilization with the small and highly charged hexametaphosphate (HMP) also potentiated the delivery of siRNA: PEI-HMP delivered GFP-specific siRNA in cell culture leading to >80% suppression in reporter gene expression (Patnaik, Arif, Pathak, Kurupati, et al., 2010). Bifunctional polyethyeneglycol PEG-bis–Phosphates played a dual role as electrostatic crosslinker while shielding acylated PEI-siRNA complexes toward the outer medium. Silencing efficiency of PEI nanoparticles was found to be comparable to commercially available transfecting agents but with reduced cytotoxicity (Nimesh & Chandra, 2009).

6.2.4 Hydrophobic and Aromatic Modifiers

Chemical methods used to increase the binding of the small siRNA molecules to PEI consist in the synthesis of prestructured polymer backbones. Introduction of hydrophobic moieties induces an auto-organization of the polymer into nanostructures ranging from micelles (mainly intramolecular hydrophobic bonds; polymeric micelles) to gel-like aggregates (interchain connections). The simplest modifications resides in the introduction of acyl (Aliabadi et al., 2011) or alkyl side chains, from C4 up to larger "fatty" hydrocarbon chains and sterol compounds (cholesteryl-PEI) (Furgeson, Cohen, Mahato, & Kim, 2002). B-PEI modified with oleyl

(C18:1) or stearic groups (C18:0) increased siRNA condensation up to threefold as compared to the parent PEI reaching over 90% of the cells after 24 h (Alshamsan et al., 2009). Similar fatty-PEIs derivatives have also successfully been used in gene transfection assays (Doody, Korley, Dang, Zawaneh, & Putnam, 2006; Furgeson et al., 2002; Han, Mahato, & Kim, 2001).

Low molecular weight polyethylenimine (PEI 1.8 kD) conjugated to deoxycholic acid (sterol) showed significantly higher siRNA delivery than unmodified B-PEI 25 kDa. The capability of forming a micelle-like inner structure enables the conjugates to encapsulate and dissolve hydrophobic anticancer drug paclitaxel. It was used for simultaneous delivery of an anticancer drug paclitaxel and siRNA forming stable ternary complexes (Jang et al., 2012).

The hydrophobic interactions that govern in these amphiphilic PEIs can be even further enhanced by additional π-π interactions of aromatic moieties, allowing an even more subtle control of the hydrophobic/hydrophilic balance. This concept can be exemplified by the very elegant work from G. Zuber: in a first generation aromatic aminoacids phenylalanine and tyrosine were coupled to B-PEI. These modifications potentiated the delivery of siRNA by PEI by 50-fold, now active at low nanomolar concentrations of siRNA (Creusat & Zuber, 2008). In a second optimization step the same group enlarged the concept of hydrophobic modifiers to self-buffering aromatic groups exemplified by pyridylthiourea-grafted polyethylenimine (πPEI; see Figure 9.2) (Creusat et al., 2012; Pinel et al., 2014).

In retrospect even plain phenol group, present in the first generation tyrosine-PEIs, is expected to contribute favorably to the buffer capacity of PEI.

Similar siRNA-optimized hydrophobic modifications of PEIs include tetramethylguanidinium chloride-hexanoic acid substituted PEI (TH-PEI) (Mahato, Kumar, & Sharma, 2013). Other aromatic modifiers enhancing the buffer potential of the resulting polymer include imidazolyl moieties (Swami, Aggarwal, et al., 2007) and histidyl residues (*N*-acryloyl-histidine) (Figure 9.2) (Bertrand et al., 2011).

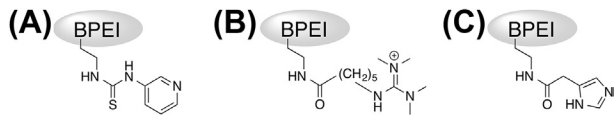

Figure 9.2 Modified PEIs with buffer potential. (A) Pyridylthiourea-grafted (πPEI), (B) Tetramethylguanidinium chloride-hexanoic acid substituted PEI (TH-PEI), (C) Imidazolyl-PEI.

7. CONCLUSION

Polyethylenimines belong to the most studied nonviral vectors for nucleic acid delivery. The mechanism of transfection of this family of polymers has been nearly unraveled. And the "high" transfection efficiency of PEI has allowed its utilization in several clinical trials. Although the results of these assays are interesting, they also underline that further improvements are necessary in order to enhance the therapeutic efficacy of the nucleic acid-based formulations. To confer new properties to PEIs (reduced cytotoxicity, prolonged blood circulation, cell targeting) a plethora of innovative modifications of the polymer have been explored. However, when developing new PEI-based vectors, one has to keep in mind that an increase of the complexity of the delivery vehicle construct raises exponentially the difficulties in order to respect quality standards needed for clinical trials.

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CHAPTER TEN

Bioresponsive Polymer-Based Nucleic Acid Carriers

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Contents

Abstract

Nucleic acid carriers need to possess multifunctionality for overcoming biological barriers, such as the stable encapsulation of nucleic acids in extracellular milieu, internalization by target cells, controlled intracellular distribution, and release of nucleic acids at the target site of action. To fulfill these stepwise functionalities, "bioresponsive" polymers that can alter their structure responding to site-specific biological signals are highly useful. Notably, pH, redox potential, and enzymatic activities vary along with microenvironments in the body, and thus, the responsiveness to these signals

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enables to construct nucleic acid carriers with programmed functionalities. This chapter describes the design of bioresponsive polymers that respond to various biological microenvironments for smart nucleic acids delivery.

1. INTRODUCTION

Nucleic acids, including plasmid DNA (pDNA), antisense oligonucleotide (ASO), and small interfering RNA (siRNA), can regulate the gene expression within cells in a sequence-specific manner (Kajimoto, Sato, Nakamura, Yamada, & Harashima, 2014; Kanasty, Dorkin, Vegas, & Anderson, 2013; Ling, Fabbri, & Calin, 2013). This property makes nucleic acids highly attractive not only as biological research tools but also as potential drugs for treating refractory diseases, such as Alzheimer disease, acquired immune deficiency syndrome (AIDS), and cancer (Daka & Peer, 2012; Draz et al., 2014; Kole, Krainer, & Altman, 2012). The discovery of RNA interference (RNAi) in 1998 (Fire et al., 1998) and the completion of human genome analysis in 2003, two noteworthy accomplishments, have encouraged researchers to pursue nucleic acid therapeutics, as reflected by more than 10,000 reports per year related to siRNA. Nevertheless, several fundamental issues need to be resolved in nucleic acid therapeutics, such as the rapid enzymatic degradation of nucleic acids in extracellular milieu (or outside the target site) and the inefficient cellular internalization of nucleic acids owing to their electrostatic repulsion with anionic cellular membranes. Therefore, appropriate delivery methodologies need to be developed to overcome the poor bioavailability of nucleic acids.

Various types of materials, including lipids, polymers, and inorganic nanoparticles, have been designed for use as the delivery vehicles of nucleic acids (Guo & Huang, 2012; Kanasty, Whitehead, Vegas, & Anderson, 2012; Lee & Kataoka, 2009; Nakase et al., 2012; Nishiyama & Kataoka, 2006; Wagner, 2012). The polymer-based nanocarriers, termed polyplexes, are among the most promising formulations. Polyplexes can be readily prepared in aqueous solutions by simply mixing polycations with oppositely charged nucleic acids. Polyplex formation dramatically enhances the stability of nucleic acids against enzymatic degradation and further facilitates the cellular uptake of payloads by eliminating electrostatic repulsion with the cellular surface (Jhaveri & Torchilin, 2014; Miyata, Nishiyama, & Kataoka, 2012; Wagner, 2012). Most importantly, polymers can be made to have various functional units or segments in a single molecule, providing polyplexes with desired functionalities (Duncan, Ringsdorf, & Satchi-Fainaro, 2006; Miyata et al., 2012). Notably, numerous reports have focused

on the functionalization of polymers and polyplexes for higher transfection efficiency with lower cytotoxicity (Jhaveri & Torchilin, 2014; Miyata et al., 2012; Wagner, 2012). In this regard, the combination of functional units or segments generates immense possibilities in polymer design, as demonstrated by the combinatorial approach (Anderson, Lynn, & Langer, 2003; Siegwart et al., 2011). Thus, a rational polymer design strategy is demanded for developing of smart polyplexes, directed toward the successful delivery of nucleic acids to target sites.

The important criteria in polymer and polyplex designs are the stable encapsulation of nucleic acids within polyplexes in extracellular milieu, the selective delivery to the target cells, the controlled intracellular trafficking, the effective release of nucleic acids at the intracellular target sites, and negligible cytotoxicity and immunogenicity (Figure 10.1). Among them, the stable encapsulation and the effective release of nucleic acids are apparently conflicting. A sophisticated answer to this dilemma in delivery functions is to construct smart polyplexes using "bioresponsive" polymers that fulfill the programmed function in response to site-specific biological signals. In this regard, pH, glutathione (GSH) and adenosine triphosphate (ATP), and enzymatic activities are known to vary considerably according to the biological microenvironment (Alarcon, Pennadam, & Alexander, 2005; Fleige, Quadir, & Haag, 2012; Kelley, Albert, Sullivan, & Epps, 2013; Zelzer, Todd, Hirst, McDonald, & Ulijn, 2013): thus, they can be used as site-specific signals. In this chapter, we describe the rational design of bioresponsive

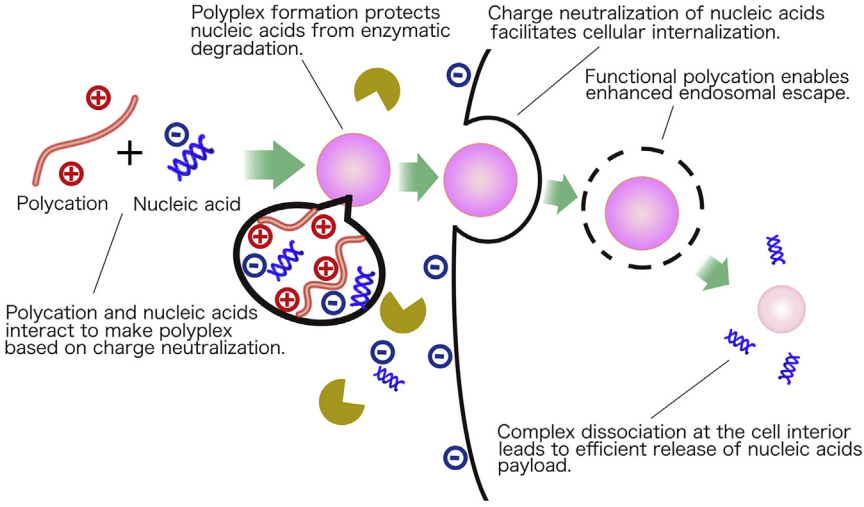


Figure 10.1 ***Strategies for efficient nucleic acids delivery based on polyplex formation.*** (See the color plate.)

polymers and their polyplexes associated with noticeable biological signals, presenting their strong potential for nucleic acid delivery.

2. POLYMER SELF-ASSEMBLIES FOR NUCLEIC ACID DELIVERY

2.1 Polyplexes Prepared with Polycations

The natural forms of nucleic acids rapidly degrade in extracellular milieu containing abundant DNases and RNases, resulting in the loss of biological (or therapeutic) activity. To have therapeutic benefits, the nucleic acids need to be protected from such enzymatic degradation until they reach the target sites, e.g., the cytoplasm for siRNA and the nucleus for pDNA. For this purpose, polycations are used to form polyplexes (or polyion complexes) with oppositely charged nucleic acids through electrostatic interactions and charge neutralization. Such complexation segregates the nucleic acids from the external environment and protects them from enzymatic attacks. Moreover, polyplexes can accelerate the cellular internalization of nucleic acids by eliminating the electrostatic repulsion between the nucleic acid payloads and the negatively charged cellular membrane (Jhaveri & Torchilin, 2014; Miyata et al., 2012; Wagner, 2012).

Many previous studies have shown that the delivery efficacy of polyplexes strongly depends on the comprised polycation structure. The molecular weight (MW) of polycations is well known to greatly affect the physicochemical properties and biological activities of polyplexes. Polycations with high MW (or more cationic moieties) can more tightly bind to negatively charged biological components, including nucleic acids and cellular membranes, through greater multisite binding. Accordingly, these polycations permit the formation of more stable polyplexes and the stronger adsorption of polyplexes on the cellular surface toward adsorptive endocytosis. However, the strong binding capacity of polycations with high MW more likely leads to an inefficient payload release at the target site and significant cytotoxicity by perturbing the integrity of anionic cellular components, e.g., mitochondrial membranes (Larsen et al., 2012). On the contrary, polycations with a low MW result in unstable polyplex formation and lower cytotoxicity because of their reduced electrostatic interactions with negatively charged substances (Fischer, Li, Ahlemeyer, Krieglstein, & Kissel, 2003). The MW of polycations can be adjusted to some extent for the desirable polyplex stability, as well as negligible cytotoxicity; a more sophisticated approach is to install an intracellularly cleavable linker into polycations for the construction of smart polyplexes with

reversible stability. In this manner, polyplexes can be stabilized in extracellular milieu by strong electrostatic interactions or covalent cross-linking and destabilized via the degradation of the polycations or cleavage of the cross-links triggered by intracellular specific signals.

After systemic administration, polyplexes encounter abundant blood components, such as albumin, antibodies, and complements, and may be captured by the reticuloendothelial systems (RES) (Alexis, Pridgen, Molnar, & Farokhzad, 2008). These biological systems recognize polyplexes as foreign bodies and rapidly eliminate them from the bloodstream toward metabolic pathways. To avoid foreign body recognition, a biological inertness is essential for polyplexes in terms of blood compatibility, as well as reduced immunogenicity. To this end, various hydrophilic polymers, including naturally occurring polysaccharides and synthetic polymers, have been conjugated to polycations to provide polyplexes with a biologically inert surface, the so-called "stealth" ability (Allen & Chonn, 1987; Gref et al., 1994; Kataoka, Kwon, Yokoyama, Okano, & Sakurai, 1993; Klibanov, Maruyama, Torchilin, & Huang, 1990). Poly(ethylene glycol) (PEG) is a golden standard polymer for the stealth ability because of its nonionic, hydrophilic, and flexible natures; PEG chains on the polyplex surface can suppress the access of blood components because of its steric repulsive effect (Harris et al., 2003; Knop, Hoogenboom, Fischer, & Schubert, 2010). Note that the biocompatibility of PEG has been widely accepted because several formulations of PEG-modified (PEGylated) drugs have been already approved by the US Food and Drug Administration (FDA), e.g., Doxil® (PEGylated liposomal doxorubicin) for the treatment of cancer, Pegasys® (PEGylated interferon) for the treatment of chronic hepatitis C, and Macugen® (PEGylated aptamer) for the treatment of age-related macular degeneration. On the other hand, the PEGylation of polyplexes concurrently suppresses the polyplex adsorption on the target cellular surface, resulting in an inefficient cellular uptake. The introduction of biodegradable linkers between PEG and polycations is a feasible solution to the PEG dilemma because the linkers can selectively liberate PEG from the polyplex surface at the target tissues. Another promising solution is to use ligand molecules that specifically bind to the overexpressed molecule on the target cellular surface. Notably, various ligand molecules, e.g., lactose and galactose for liver targeting and transferrin and the cyclo-arginine-glycine-glutamic acid (cRGD) peptide for cancer targeting, have been installed into the distal end of PEG as the targeting moiety (Bae & Kataoka, 2009; Cabral & Kataoka, 2014).

2.2 Polyplex Micelles Prepared with Block Copolymers

To fulfill the aforementioned criteria in polymer and polyplex designs, block copolymers of PEG and polycations have been developed as a platform material for the construction of PEGylated polyplexes (polyplex micelles) (Figure 10.2(A)) (Christie, Nishiyama, & Kataoka, 2010; Kakizawa & Kataoka, 2002; Kataoka et al., 1993, 1996, 2001; Katayose & Kataoka, 1997). Polyplex micelles comprise the polyion complex core and the surrounding PEG corona, with a size of 30–100 nm and narrow size distribution (Chen et al., 2012; Matsumoto et al., 2009; Tockary et al., 2013). This size range protects small nucleic acids, such as ASO and siRNA, from rapid renal excretion. Note that the threshold for renal excretion is reported at MW ~40,000 for hydrophilic polymers (Bohrer et al., 1978; Fox, Szoka, & Fréchet, 2009; Yamaoka, Tabata, & Ikada, 1994) and ~6 nm for spherical solid nanoparticles (Choi et al., 2007, 2009; Lee et al., 2012). As a result, systemically administered polyplex micelles can preferably accumulate in solid tumors through the leaky vasculature network and the immature lymphatic drainage in tumor tissues, termed the enhanced permeability

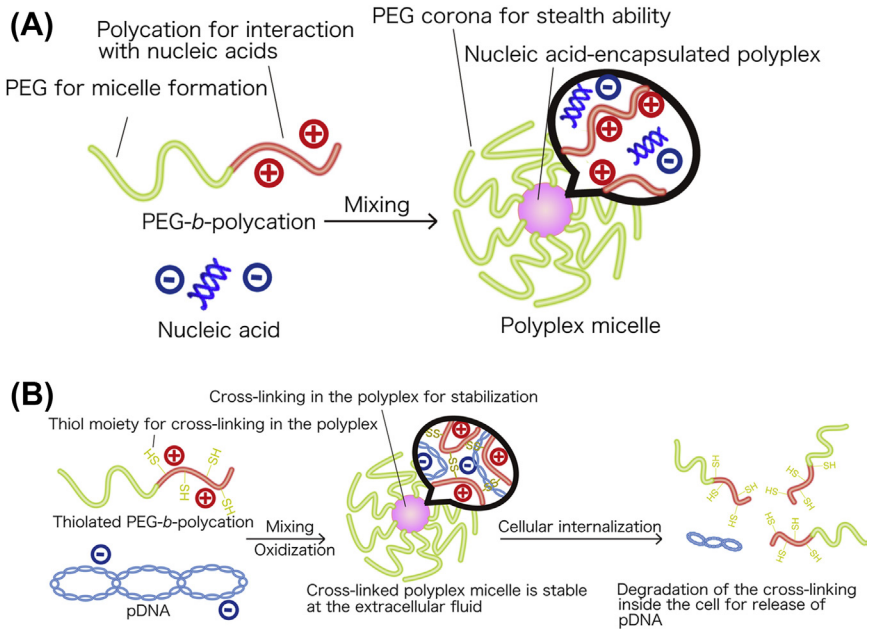


Figure 10.2 ***Polyplex micelle formation using PEG-b-polycation (A), and bioresponsive polyplex micelle based on intracellularly cleavable disulfide linkage (B).*** Details are described in Section 3.3.1.

and retention (EPR) effect (Maeda, Wu, Sawa, Matsumura, & Hori, 2000; Matsumura & Maeda, 1986). Most importantly, the PEG corona plays a pivotal role in maintaining the nanoscale size of polyplex micelles in the bloodstream. Polyplexes without PEG (or stealth ability) most likely form larger aggregates through the adsorption of blood components or the salting-out effect, although their initial size in a buffer solution is less than 100 nm (Kakizawa & Kataoka, 2002; Takae et al., 2008). Notably, intravital confocal videography has enabled the direct observation of microscale aggregate formation from polyplexes prepared with Cy5-labeled pDNA and polyethyleneimine (PEI) or polylysine (PLys) in the absence of PEG (Nomoto et al., 2011). Apparently, the Cy5 signals were not homogenously dispersed within the blood vessels but displayed large spots, similar to platelets stained with a fluorescent antibody (upper and middle panels in Figure 10.3). In sharp contrast, the polyplex micelles prepared with the

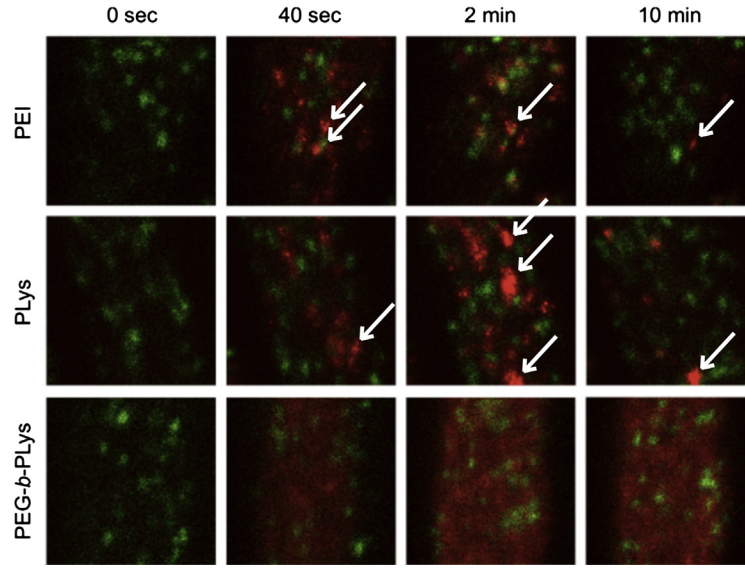


Figure 10.3 ***Representative intravital confocal videographs of homopolymer polyplexes and polyplex micelles circulating in the bloodstream of a mouse earlobe (image size: ~80 × ~80 μm).*** Homopolymer polyplexes were prepared with Cy5-labeled pDNA and PEI (22 kDa) or PLys (4–15 kDa). Polyplex micelles were prepared with Cy5-labeled pDNA and PEG-*b*-PLys (12 kDa for PEG and 7 kDa for PLys). Platelets were stained with DyLight 488-labeled antibody prior to observation. The fluorescence signals of Cy5 and DyLight 488 are shown in red and green, respectively. Arrows indicate the representative microscale aggregates. This figure is reproduced with permission from Nomoto et al. (2011). (See the color plate.)

Cy5-labeled pDNA and PEG-*b*-PLys successfully avoided such aggregate formation because their Cy5 signals had spread over the blood vessels (lower panels in Figure 10.3). Block copolymers can be further elaborated to have bioresponsive functionalities by installing intracellularly cleavable linkers into the polycation main chain, or side chains, and the junction of PEG and polycation, as illustrated in Figure 10.2(B) (Christie et al., 2012; Miyata et al., 2004; Suma, Miyata, & Anraku, 2012; Takae et al., 2008). Eventually, smart polyplex micelles prepared with such bioresponsive block copolymers have achieved the tumor-targeted delivery of therapeutic pDNA or siRNA through systemic administration, eliciting significant gene expression or gene silencing, respectively, directed toward significant antitumor activity (Christie et al., 2012; Vachutinsky et al., 2011). Representative bioresponsive block copolymers are described in the sections as follows.

3. REDUCTIVE ENVIRONMENT-RESPONSIVE NUCLEIC ACID DELIVERY

Redox potentials in the body are strictly regulated according to the sites and the conditions (Banerjee, 2012; Kemp, Go, & Jones, 2008). Three molecules, GSH, thioredoxin (Trx), and cysteine (Cys), are known to mainly regulate the redox potential (Circu & Aw, 2011; Kemp et al., 2008). GSH is a tripeptide consisting of glutamic acid, cysteine, and glycine, having an MW of ~300 Da. Trx is a class of small redox enzymes with an MW of ~12,000 Da. Note that the redox potential is not simply determined by the concentration of the reducing molecules but calculated with the Nernst equation.

$$E = E_0 + (RT/nF) \times \ln\left([\text{oxidized reductant}]/[\text{reductant}]^x\right),$$

where E_0 is the standard half-cell potential, R is the gas constant, T is the absolute temperature, n is the number of moles, F is the Faraday's constant, and x is 2 for GSH and Cys and 1 for Trx (Go & Jones, 2010; Schafer & Buettner, 2001). The redox potential is defined for each reducing molecule, and the lower values correspond to greater reducing activity.

The disulfide bond (–SS–) has been extensively highlighted as a reversible linkage responding to the redox potential (Saito, Swanson, & Lee, 2003). Disulfide formation arises via the reaction between two thiol groups. Disulfide linkages, while stable under oxidizing conditions, can be cleaved under reducing conditions. Notably, disulfide linkages in proteins are well known

to be cleaved under the reductive cytosol, whereas they are stable in less reductive extracellular fluids (Cumming et al., 2004; Rietsch & Beckwith, 1998). Thiol moieties or disulfide linkages can be introduced into polymers to develop of reductive environment-responsive polymers (Christie et al., 2011; Kakizawa, Harada, & Kataoka, 1999; McKenzie, Kwok, & Rice, 2000; Miyata et al., 2004). In this section, we describe the various reductive environments in the body and representative reductive environment-responsive polymers and polyplexes.

3.1 Various Biological Reductive Environments

The blood concentration of GSH is around at 1–10 μM, generating a moderately reductive environment. The slightly variable GSH concentrations are ascribed to the metabolism affected by aging and the daily lifestyle (Kemp et al., 2008; Moriarty-Craige & Jones, 2004). For example, aging is associated with the decreasing the concentration of reduced GSH, as well as the increasing concentration of the GSH dimer (GSSG), i.e., blood oxidization. In humans, the redox potential of GSH, i.e., E(GSH), is maintained at the same level until 45 years old, followed by an increase of 0.7 mV per year (Jones, Mody, Carlson, Lynn, & Sternberg, 2002). With regard to the lifestyle, smoking leads to blood oxidation; smoking products, e.g., acrolein and reactive oxygen species, oxidize GSH to GSSG. The plasma GSH concentration in humans older than 50 years is reported to be 1.8 μM for smokers and 2.4 μM for nonsmokers (Moriarty et al., 2003). In addition, excessive alcohol consumption affects the liver to decrease the amount of GSH secreted from the liver to the blood.

The GSH concentration in the cytosol is drastically elevated up to 1–10 mM, which is 1000 times higher than that in the bloodstream, generating highly reductive conditions (Meister & Anderson, 1983; Saito et al., 2003). In detail, 85–90% of the intracellular GSH is distributed in the cytosol, 10–15% resides in the mitochondria, and the remaining reside in the nucleus and the endoplasmic reticulum (Hwang, Sinskey, & Lodish, 1992; Jocelyn & Kamminga, 1974; Meredith & Reed, 1982; Wu, Fang, Yang, Lupton, & Turner, 2004). Table 10.1 summarizes the variable redox potentials of GSH, Trx, and Cys within a cell. The mitochondrial compartment exhibits the lowest redox potential, thereby being most reductive within the cell. Considering that mitochondria consume oxygen and produce radical oxygen species via aerobic respiration, they require a stronger reductive condition for minimizing oxidative damages (Raza, 2011). The redox potential in the nuclei, which is believed to be regulated mainly by Trx, is

Table 10.1 Divergent redox potentials of GSH, Trx, and Cys within a cell (Kemp et al., 2008)

	Cytosol	Mitochondria	Nucleus	Endoplasmic reticulum
E(GSH)	−260 mV	−300 mV	N.R.	−150 mV
E(Trx)	−280 mV	−340 mV	−300 mV	N.R.
E(Cys)	−160 mV	N.R.	N.R.	N.R.

"N.R." denotes "Not Reported"

Table 10.2 Redox potentials of GSH responding to the cell cycle (Circu & Aw, 2011; Kemp et al., 2008)

	Proliferation	Differentiation	Apoptosis	Necrosis
E(GSH)	−260 mV	−220 mV	−170 mV	−150 mV

lower than that in the cytosol (Kemp et al., 2008). In contrast, the redox potential in the endoplasmic reticulum is moderate, and most of the GSH exists in the oxidized state (GSSG) (Hwang et al., 1992). Note that the intracellular reductive environment is affected by the cell cycle. As summarized in Table 10.2, proliferating cells possess the lowest redox potential in the cytosol, followed by differentiating, apoptotic, and necrotic cells (Banerjee, 2012; Dobbelsteen et al., 1996; Jones et al., 1995, 2010; Markovic et al., 2009).

3.2 Stability of the Disulfide Linkage in the Body

As described in Section 3.1, the bloodstream maintains a moderate reductive environment, although there are slight differences because of age and lifestyle. For using the disulfide linkages as a reductive environment-responsive linker, knowing their stability in the bloodstream, as well as the cytosol, is important. A dextran–peptide conjugate via disulfide linkage (Dex-SS-Pep, MWs of Dex and Pep are approximately 25,000 Da and 673 Da, respectively) was reported to liberate 30, 60, and 80% of the peptide after 2, 4, and 6 h of incubation, respectively, in an aqueous buffer (pH 7.2) containing 2 μM GSH (corresponding to the GSH concentration in the blood) at 37 °C (Shahnaz et al., 2012). The peptide liberation from the conjugate was attributed to the thiol-disulfide exchange between Dex-SS-Pep and GSH, suggesting a gradual cleavage of the disulfide linkages in the bloodstream. The stability of disulfide linkages was also investigated on the PEG palisade on a liposomal surface (Ishida, Kirchmeier, Moase, Zalipsky, & Allen, 2001). A disulfide conjugate of PEG and distearoylphosphatidylethanolamine

(DSPE) (PEG-SS-DSPE) and a control without disulfide linkage (PEG-DSPE) were used to prepare doxorubicin-loaded PEGylated liposomes. When these PEGylated liposomes were intravenously injected into mice, the remaining amounts in the blood were 1 and 10% at 12 h after injection for liposomes with PEG-SS-DSPE and PEG-DSPE, respectively. These results suggest that the PEGylated liposome with PEG-SS-DSPE more rapidly destabilized in the blood compared with the control without disulfide linkages, presumably because of the detachment of the liposomal surface PEG via disulfide cleavage. The loss of surface PEG chains might compromise the stealth ability of PEGylated liposomes, leading to recognition by the biological defense systems (e.g., RES) and elimination from the blood (Alexis et al., 2008). Altogether, the reductive environments in the blood (or extracellular fluid) were demonstrated to be weak but significant. Thus, for systemic nucleic acid delivery, the bioresponsive polymers containing disulfide linkages should be designed to avoid the rapid structural change through the disulfide cleavage in the bloodstream, particularly by engineering thiol-enriched polycations or polyanions for preparing multiple disulfide linkages inside the polyplexes (sections 3.3.1 and 3.3.2)

The disulfide cleavage has been reported to proceed in the cytosol within several minutes, whereas it took several hours in the bloodstream (Wu et al., 2013). A previous study examined the kinetics of the disulfide cleavage at 5 mM dithiothreitol (DTT), using a compound that can fluoresce after the cleavage of the contained disulfide linkage. The increase in the fluorescence intensity reached a plateau after 15 min, indicating that the disulfide cleavage was completed under reductive conditions. Moreover, the same compound, when added to the cell culture medium, generated fluorescence within cultured cells. In contrast, the treatment of *N*-ethylmaleimide, which is known to react with intracellular thiol compounds through Michael addition, inhibited the generation of fluorescence in the cells, demonstrating that intracellular thiol compounds were required for the disulfide cleavage within the cells.

3.3 Reductive Environment-Responsive Polyplexes

3.3.1 Disulfide Cross-Linked Polyplex Micelles

Colloidal polyplexes are considered to be in a dynamic state in aqueous media. They are likely to be disintegrated in the presence of competitive polyanions via counter polyanion exchange; competitive polyanions can interact with the polycation segment in a polyplex, possibly excluding the

nucleic acid payloads from the polyplex (Palte & Raines, 2012; Zheng et al., 2012). Anionic extracellular matrices are a potential destabilizing factor for circulating polyplexes after systemic administration. In particular, strongly anionic glycocalyx, such as heparan sulfate, present in the extracellular matrices of glomerular basement membranes, is reported to be responsible for the dissociation of siRNA-loaded polyplexes in the bloodstream, leading to their rapid elimination from the circulation (Zuckerman, Choi, Han, & Davis, 2012). Thus, the reversible cross-linking of the dynamic polyplex structures with disulfide linkages is a promising approach for suppressing the dissociation, enabling their longevity in the bloodstream. Notably, we have prepared disulfide cross-linked polyplex micelles using PEG-*b*-PLys modified with thiol moieties in PLys side chains (Figure 10.2(B)) (Christie et al., 2012; Miyata et al., 2004; Vachutinsky et al., 2011). The obtained cross-linked micelles were significantly stabilized against the dissociation induced by competitive polyanions in the absence of reducing agents, whereas they facilitated the payload release in the presence of DTT or GSH (Matsumoto et al., 2009; Miyata et al., 2004; Oe et al., 2014). Consequently, the cross-linked micelles showed a 10-fold increase in the pDNA transfection efficiency compared with the noncross-linked controls in cultured cells (Miyata et al., 2004). Furthermore, in the cross-linked micelles, when intravenously injected into mice, ~30% of the dose remained in the collected blood, whereas ~10% of that was detected in the noncross-linked controls (Vachutinsky et al., 2011). Such improvements demonstrate the utility of disulfide cross-linking for systemic nucleic acid delivery. In addition to the disulfide cross-linking, cRGD peptide, which specifically binds to the αvβ3/αvβ5 integrins that are overexpressed on the surface of various cancer and tumor-related endothelial cells (Arap, Pasqualini, & Ruoslahti, 1998; Millard, Odde, & Neamati, 2011), can be concurrently installed as a tumor-targeting ligand on the micellar surface using an acetal-bearing PEG at the distal end (Oba et al., 2007). Notably, such actively targeted cross-linked micelles successfully enhanced the tumor accumulation of the payloads in subcutaneous tumor models, such as pancreatic cancer (BxPC3), cervical cancer (HeLa), and lung cancer (A549), through systemic administration. Finally, a significant antitumor activity was obtained by functionalized micelles incorporating the pDNA coding soluble fms-like tyrosine kinase-1 (sFlt-1), which is a splice variant of the vascular endothelial growth factor (VEGF) receptor-1, and siRNAs targeted for VEGF and VEGF receptor-2, directed toward antiangiogenic cancer therapies (Christie et al., 2012; Vachutinsky et al., 2011).

3.3.2 Reversibly Polymerized Nucleic Acids

Not only polycations but also nucleic acids can be directly functionalized using disulfide linkages for the reversible polyplex stability. Small nucleic acids can be chemically synthesized to possess reactive groups, such as amine, thiol, azide, and biotin, at their termini, i.e., 3′- and 5′-ends (Jeong, Mok, Oh, & Park, 2009; Singh, Murat, & Defrancq, 2010). In particular, the nucleic acids modified with thiol groups at both termini can generate reversibly polymerized nucleic acid derivatives based on the formation of disulfide linkages (Figure 10.4(A)) (Lee et al., 2010; Mok, Lee, Park, & Park, 2010). The increased anionic charges per molecule via the polymerization elicit greater electrostatic interactions with polycations for enhancing the polyplex stability, whereas the enhancement in stability is compromised via the disulfide cleavage in the reductive cytosol for the payload release. For instance, covalently conjugated monomeric siRNA via the disulfide linkage (i.e., multimerized siRNA) was reported. When mixed with PEI, multimerized siRNA-loaded polyplexes exhibited a stronger gene silencing effect both in vitro and in vivo compared with monomeric siRNA-loaded polyplexes. Note that the polyplex-mediated gene silencing effect was similar between the dimeric siRNA and multimerized siRNA, suggesting that 80 anionic charges in the dimeric siRNA enabled the stable polyplex formation with PEI.

To more precisely control the polymerized structure of siRNA, several groups including ours have reported siRNA–polymer conjugates using chemically modifiable siRNAs (Rozema et al., 2007; Takemoto et al., 2010, 2013).

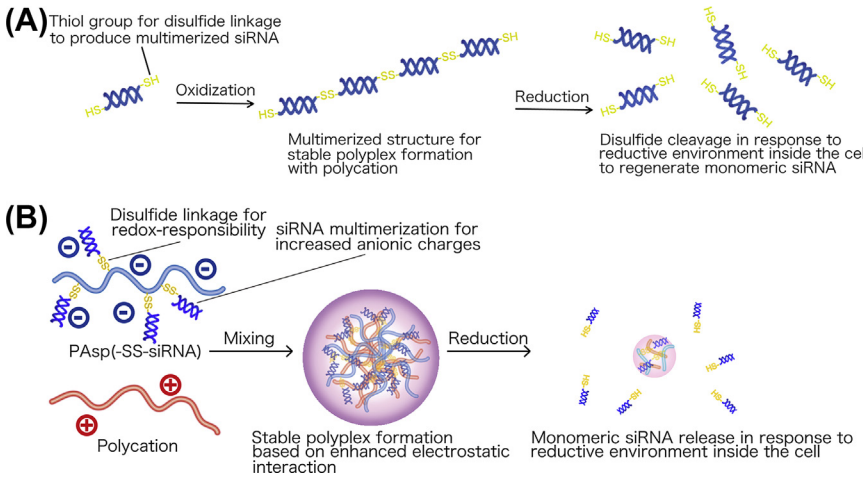


Figure 10.4 ***Multimerized siRNA (A) and siRNA-conjugate design (B) based on disulfide linkage.***

siRNA–polymer conjugation can amplify the inherent properties of siRNA and also endow siRNA with new functionalities. In this regard, we have developed an siRNA–polymer conjugate that is a poly(aspartic acid) derivative bearing four siRNA pendant groups in the side chains through disulfide linkages (PAsp(-SS-siRNA)) (Figure 10.4(B)) (Takemoto et al., 2010). The PAsp(-SS-siRNA) enabled more stable complexation with polycations compared with monomeric siRNA in the presence of counter polyanions, while released monomeric siRNA after the addition of reducing agents. Consequently, PAsp(-SS-siRNA)-loaded polyplexes elicited a five fold increase in the cellular uptake of siRNA and significantly greater gene silencing activity in cultured cancer cells, presumably because of the higher stability in the cell culture media. The enhanced stability of PAsp(-SS-siRNA) polyplexes was further demonstrated in a blood circulation test. The PAsp(-SS-siRNA)-loaded polyplex micelles prepared with PEG-*b*-PLys showed substantially longer blood circulation (half-life ~50 min) compared with the monomeric siRNA-loaded polyplex micelles (half-life ~10 min).

4. ACIDIC PH-RESPONSIVE NUCLEIC ACID DELIVERY

Blood pH is steadily regulated at around 7.4 mainly because of the buffering effect of carbonic acids (Casey, Grinstein, & Orlowski, 2010). Carbon dioxide produced by aerobic respiration in mitochondria is converted to bicarbonate ion through spontaneous dissolution in biological fluids, including the cytosol and blood, or the catalytic effect of carbonic anhydrase present on the cellular membrane and in the cytosol (Casey et al., 2010; Supuran, Scozzafava, & Casini, 2003). The bicarbonate ions (~25 mM) regulate the blood pH by collaborating with other ionic moieties, such as phosphate and ionic residues of amino acids (Casey et al., 2010; Roos & Boron, 1981), whereas the cytosolic pH (i.e., ~7.2) is slightly acidic compared with the blood pH (Srivastava, Barber, & Jacobson, 2007). The slightly lower pH is partly attributed to numerous acidic metabolites residing in the cytosol, e.g., adenosine triphosphate (ATP). The ATP concentration in the cytosol (1–10 mM) is 10-fold higher than that in the extracellular milieu (0.4 mM) (Gorman, Feigl, & Buffington, 2007; Gribble et al., 2000; Traut, 1994). In contrast, several abnormal tissues and intracellular organelles generate acidic microenvironments. Hypoxic regions, such as ischemic tissues and deeper tumor tissues, where anaerobic respiration is substantially activated, produce more acidic metabolites and decrease the extracellular pH (e.g., ~6.7 in tumor tissues) (Gatenby & Gillies, 2004; Helmlinger, Yuan, Dellian, & Jain,

1997; Jain & Stylianopoulos, 2010). In addition, endosome and lysosome are well-known intracellular organelles possessing acidic luminal pHs. Macromolecular substances internalized by the cells via endocytosis are segregated within the endosomal vesicle, where the luminal pH decreases from 7.4 to 5 via the maturation, followed by fusion with the lysosome with the luminal pH of ~4 (Jentsch, 2008; Luzio, Pryor, & Bright, 2007; Weisz, 2003). Thus, the functional moieties that respond to the aforementioned pH can be installed into the polymer structure for designing pH-responsive nucleic acids carriers. The following section describes various pH conditions in the body in more detail and the designs of acidic pH-responsive polymers and their polyplexes.

4.1 Various pH Conditions

Intracellular pHs significantly vary along with organelles (Figure 10.5), which are segregated from the cytosol by the lipid bilayer membrane. In the endosomal pathway, the luminal pH of the endosome decreases from 7.4 to 5 in association with the maturation to the late endosome and finally reaches ~4 through the fusion with the lysosome (Jentsch, 2008; Luzio et al., 2007; Weisz, 2003). Such acidic pHs are essential for activating the enzymatic hydrolysis of inclusions (Luzio et al., 2007). In the secretory pathway, the luminal pH also gradually decreases as follows: 7.2 in the endoplasmic reticulum (ER), 6.7 in the *cis*-Golgi, 6.0 in the *trans*-Golgi, and 5.2 in the secretory granules (Paroutis, 2004; Wu et al., 2001). The decrease in the pH can be explained by the variable proton permeabilities at the luminal membrane and the activated proton pumping by a vacuolar ATPase toward the luminal acidification (Casey et al., 2010; Forgac, 2007; Paroutis, 2004). The luminal protons are reported

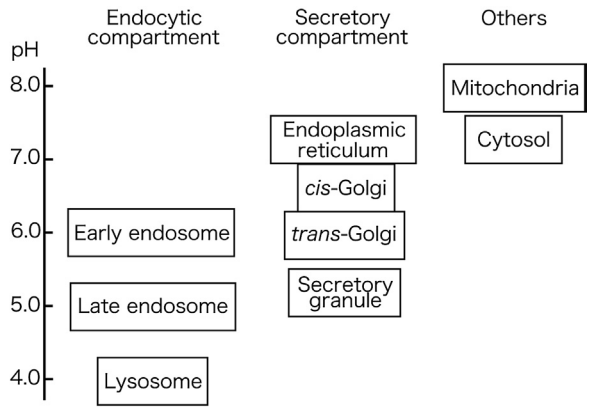


Figure 10.5 ***Various pHs within a cell.***

to readily pass through the endosomal and ER membranes; however, they are trapped in the other vesicular compartments downstream (Casey et al., 2010; Schapiro & Grinstein, 2000; Wu et al., 2001). On the contrary, the luminal pH of mitochondria is reported as slightly basic, ~8 (Abad, Di Benedetto, Magalhaes, Filippin, & Pozzan, 2004; Llopis, McCaffery, Miyawaki, Farquhar, & Tsien, 1998). Mitochondria synthesize ATP through oxidative phosphorylation, which harnesses the pH gradient across the membrane: the electron transport chain in mitochondria extrudes protons to the cytosol, leading to the basic luminal pH.

The cell cycle also affects the intracellular pH. Proliferating cells increase the cytosolic pH up to 7.4 from the basal pH of 7.2 (Pouysségur, Franchi, L'Allemain, & Paris, 1985; Putney & Barber, 2003; Webb, Chimenti, Jacobson, & Barber, 2011). The population of proliferating cells has been reported to increase by externally adjusting the intracellular pH to 7.4, where the cells were incubated in the presence of 50 mM $NaHCO_3$ and 15% CO_2, indicating a correlation between the intracellular pH and cell cycle (Putney & Barber, 2003). In addition, the intracellular pH decreases to ~6.8 when the cells undergo apoptosis (Lagadic-Gossmann, Huc, & Lecureur, 2004; Matsuyama, Llopis, Deveraux, Tsien, & Reed, 2000). The pH decrease toward apoptosis is also associated with the activation of cytochrome *c*-derived caspase, which is one of the major apoptotic roots and optimized at a pH of 6.3–6.8 (Matsuyama et al., 2000). This suggests that the intracellular pH continuously changes to regulate the cellular homeostasis. The main participant that regulates the intracellular pH during the cell cycle is the Na^+–H^+ exchanger (NHE), which harnesses the transmembrane gradient of Na^+ and concurrently exports the intracellular protons, imports extracellular Na^+, and adjusts the pH of the cell cycle (Brett, Donowitz, & Rao, 2005; Madshus, 1988; Orlowski & Grinstein, 2008; Webb et al., 2011).

Tumor tissues and cells have distinct pH characteristics from normal tissues and cells, i.e., lower extracellular pH and higher intracellular pH (Cardone, Casavola, & Reshkin, 2005; Webb et al., 2011). ATP is abnormally produced by anaerobic respiration in tumor tissues toward the acidification of the cell exterior (Gatenby & Gillies, 2004). In detail, this type of ATP production is ascribed to glycolysis and is accompanied with lactic acid production. Overexpressed membrane transporters in cancer cells progressively facilitate the efflux of protons and monocarboxylic acids, including lactic acid, resulting in the aforementioned acidic extracellular pH and basic intracellular pH in tumor microenvironments (Cardone et al., 2005; Gatenby & Gillies, 2004). The extracellular pH in tumor tissues gradually decreases depending on the distance from the blood vessel, e.g., pH 6.9, 6.8, and 6.7

at a distance of 100, 200, and 300 μm, respectively, and the intracellular pH increases up to 7.6 (Cardone et al., 2005; Helmlinger et al., 1997). The aforementioned abnormal character of the pH in tumor tissues is considered to facilitate their progression and invasion; the increased intracellular pH enables cell proliferation, and the decreased extracellular pH promotes cell invasion (Putney & Barber, 2003; Stock & Schwab, 2009).

4.1.1 Acidic pH-Responsive Protonatable Structures

Nucleic acids and their polyplexes are generally internalized by the cells via endocytosis, followed by lysosomal digestion. Therefore, to circumvent the degradation of nucleic acids, polyplexes need to be designed for delivering them from the endosome to the cytosol (i.e., endosomal escape). To this end, polycations have been developed to comprise amino groups with low pKa (5–7), enabling endosomal pH-responsive protonation. This facilitated protonation elicits the extensive influx of protons and chloride ions as counterpart, which increases the osmotic pressure within the endosome, directed toward the endosomal disruption and the subsequent release of entrapped substances to the cytosol (the so-called proton sponge effect (Boussif et al., 1995; Thomas & Klibanov, 2002)) (Figure 10.6(A)). Notably, PEI compromises the endosomal acidification (or the pH decrease in the endosome) and elevates the chloride ion concentration in the endosome, associated with 140% increase in the relative volume of the endosome (Sonawane, Szoka, & Verkman, 2003). On the other hand, the endosomal pH-responsive protonation increases the cationic charge density of polycations within the endosome, enabling stronger interactions of the polycations with the negatively charged endosomal membrane, directed toward the membrane destabilization. The direct interaction between polycations and the endosomal membrane is also the underlying mechanism for polycation-induced endosomal escape (Figure 10.6(B)) (Miyata et al., 2008; Suma, Miyata, & Ishii, 2012; Uchida et al., 2011). Altogether, both mechanisms are considered, i.e., the proton sponge effect and direct interactions with the endosomal membrane, to possibly synergistically facilitate the endosomal escape of polyplexes.

PEI is the most widely used polycation that fulfills the endosomal escape of nucleic acids (Figure 10.7(A)) (Demeneix & Behr, 2005; Jäger, Schubert, Ochrimenko, Fischer, & Schubert, 2012). Its amino groups can protonate at the endosomal acidic pH; the protonation degree is ~20% at the physiological pH of 7.4 and ~40% at pH 5.0 (Suh, Paik, & Hwang, 1994). Polyplexes prepared with PEI are demonstrated to enable several orders of magnitude higher efficiency in the gene expression of pDNA in cultured cells compared with the control

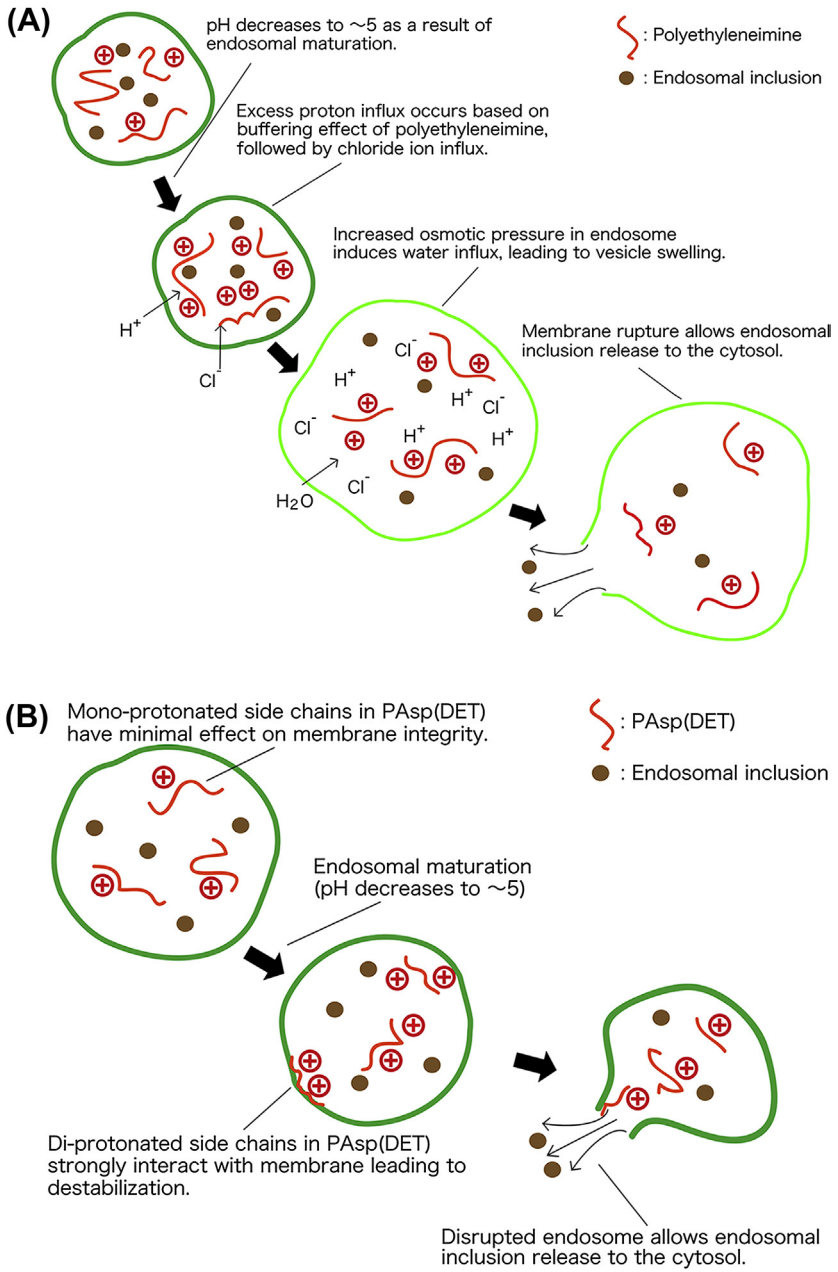


Figure 10.6 ***Proton sponge effect induced by polyethyleneimine (PEI) (A) and endosomal membrane destabilization induced by PAsp(DET) (B). These figures are reproduced with permission from (Takemoto & Nishiyama, 2014).***

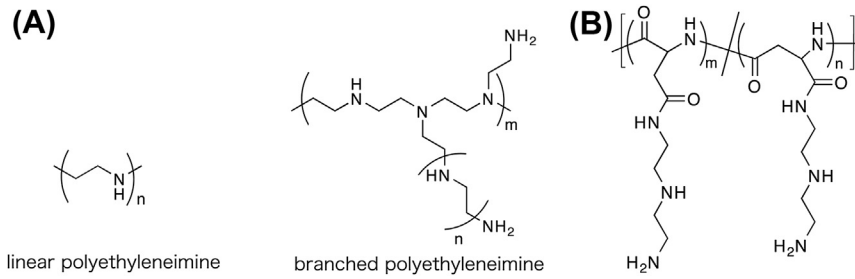


Figure 10.7 ***Chemical structures of PEI (A) and PAsp(DET) (B).***

polyplex prepared using PLys with high pKa amines (Boussif et al., 1995). However, the nondegradability of PEI often causes cytotoxicity and immune response (Fischer et al., 2003; Itaka, Ishii, Hasegawa, & Kataoka, 2010; Kunath et al., 2003). To reduce these adverse side effects, bioresponsive linkages, such as the disulfide bond (Section 3.3) and acid-labile bonds (Section 4.1.2), have been integrated into PEI, affording biodegradability for less toxic transfection (Carlisle et al., 2004; Gosselin, Guo, & Lee, 2001; Kim et al., 2005; Knorr, Ogris, & Wagner, 2008). Meanwhile, we have developed a polyaspartamide derivative (PAsp) comprising diaminoethane pendant group (termed PAsp(DET)), the amines of which have two distinct pKa's of 6.2 and 8.9 (Figure 10.7(B)) (Miyata et al., 2008). Based on these pKa values, the diaminoethane moiety in the side chains of PAsp(DET) is in the monoprotonated state at the extracellular pH of 7.4 and converts to the diprotonated state at the late endosomal pH of 5.0. The diprotonated PAsp(DET) side chains can strongly interact with the cellular membrane presumably because of the increased cationic charge density, thus destabilizing the membrane. This acidic pH-selective membrane destabilization of PAsp(DET) was demonstrated by a hemolysis assay; PAsp(DET) exhibited a potent hemolysis activity at pH 5.5, whereas a negligible hemolysis activity was observed at pH 7.4 (Miyata et al., 2008; Uchida et al., 2011). Thus, PAsp(DET) selectively destabilized the cellular membrane at the late endosomal pH for endosomal escape with minimal cytotoxicity. In addition, the polyaspartamide main chain could progressively degrade through self-catalytic reaction at neutral pH and at 37 °C. The degradability of PAsp(DET) can be explained by the underlying degradation mechanism observed in asparagine-containing peptides; the amide nitrogen at an asparagine residue makes a nucleophilic attack on the carbonyl group at the peptide bond, inducing peptide bond cleavage via succinimide formation (Figure 10.8) (Geiger & Clarke, 1987). Consequently, PAsp(DET) achieved efficient pDNA transfection without compromising the cellular homeostasis, associated with the minimal change in house-keeping

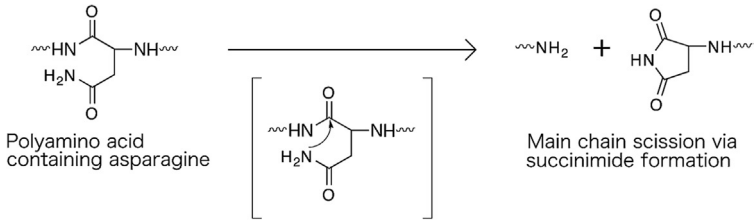


Figure 10.8 ***The mechanism of peptide cleavage reaction induced by asparagine residue.***

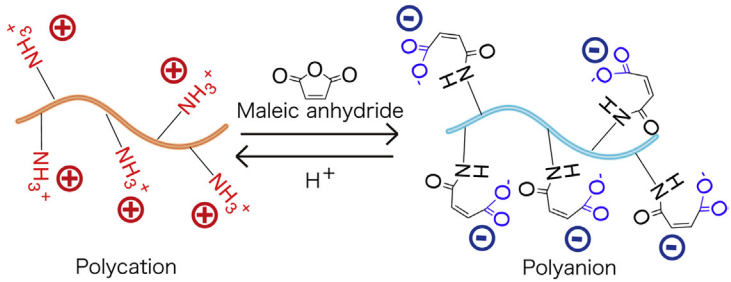


Figure 10.9 ***Charge-conversion property of MAA-introduced polymer.***

gene expression (Itaka et al., 2010). The utility of PAsp(DET) has been further demonstrated for animal disease models. Polyplex micelles prepared with PEG-*b*-PAsp(DET) and pDNA encoding sFlt-1 showed the significant growth inhibition for subcutaneous pancreatic (BxPC3) tumor in mice (Ge et al., 2014). More recently, PEG-*b*-PAsp(DET) has been tested for preparing polyplex micelles with mRNA. The obtained micelles have been confirmed to enable significant protein production at the central nervous system in the brain after intrathecal injection (Uchida et al., 2013).

4.1.2 Acidic pH-Responsive Degradable Structures

Acid-labile linkers, such as imine, orthoester, acetal/ketal, and maleic acid amide (MAA), are highly useful for constructing smart polyplexes with acidic pH-responsive degradability. Similar to the disulfide bond, the linkers have been installed into the polycation main chain, or side chains, for preparing biodegradable polyplexes and enabling the site-specific release of nucleic acids, as well as lower cytotoxicity (Cohen et al., 2011; Guk et al., 2013; Kim et al., 2005; Knorr et al., 2008). Among them, MAA and its derivatives have recently afforded interesting polymer designs because of their unique charge-conversion property, as shown in Figure 10.9. MAA is prepared by the reaction of primary amine and maleic anhydride, providing a product with anionic moieties (Dixon & Perham, 1968; Lee et al.,

2007, 2009). The obtained polyanion is relatively stable at neutral pH, while hydrolyzed to expose the primary amines at acidic pH through MAA degradation, thereby regenerating the parent polycation. The following paragraphs focus on pH-responsive polymers that use MAA derivatives for enhanced cellular internalization and endosomal escape.

The hydrolysis kinetics of MAA derivatives depend on the substituted groups at the double bond of the MAA moiety, and thus, the charge-conversion property can be modulated by altering the substituted groups. In general, MAA from dimethyl maleic acid derivatives elicits faster hydrolysis compared with MAA from monomethyl maleic acid derivatives because of the higher pKa of carboxylate in MAA (Maeda et al., 2014; Rozema, Ekena, Lewis, Loomis, & Wolff, 2003). The degradation half-life of MAA from dimethyl maleic acid was reported to be 1.5 min at pH 5.0, whereas that of citraconic acid amide (MAA from monomethyl maleic acid) was 300 min (Rozema et al., 2003).

As aforementioned, the extracellular pH in tumor tissues is more acidic compared with normal tissues and bloodstream. Thus, the MAA chemistry enables tumor tissue-specific charge-conversion reactions for changing the net charge of polyplexes from negative to positive for and accelerating the cellular internalization. For example, a nanogel particle comprising cross-linked polycations modified with an MAA derivative that has anionic zeta-potential at pH 7.4 exhibited inefficient cellular internalization (Du, Sun, Song, Wu, & Wang, 2010). In contrast, after incubation at pH 6.7, the zeta-potential of the nanogel converted to positive, leading to efficient cellular internalization, probably because of the considerable electrostatic interactions with the positively charged nanogels and anionic cellular surface.

In addition, MAA-introduced polymers can elicit efficient endosomal escape, associated with significantly decreased cytotoxicity (Lee et al., 2008; Sanjoh et al., 2010, 2012). Polyplexes prepared with polycations generally possess cationic surface charge, thereby being potentially cytotoxic, especially in the case that a large amount of polyplexes is administered. To attenuate the cationic nature of polyplexes, the MAA-modified PAsp(DET) (i.e., a polyanionic derivative) was engineered for the surface decoration of cationic polyplexes. The decorated polyplexes substantially decreased the cytotoxicity of the parent polyplexes and concurrently enhanced the endosomal escapability because of the regeneration of PAsp(DET) within the acidic endosomal compartment. Consequently, the decorated polyplexes exhibited 10-fold higher gene expression efficiency by delivering pDNA compared with nondecorated control polyplexes (Sanjoh et al., 2010).

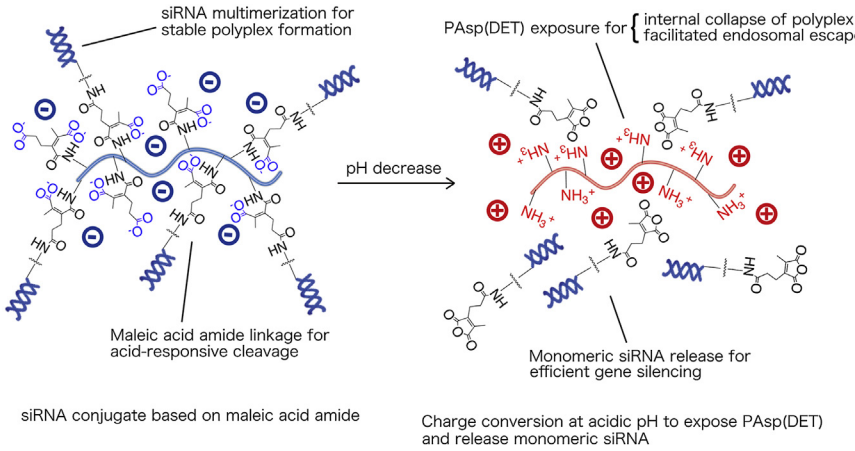


Figure 10.10 ***siRNA conjugate design utilizing MAA chemistry (Takemoto et al., 2013). This figure is reproduced with permission from (Takemoto & Nishiyama, 2014).***

The MAA chemistry can also be applied to the development of a smart siRNA conjugate equipped with pH responsibility. In a previous study, several siRNA molecules were conjugated to the side chains of PAsp(DET) via an MAA-based linker (Figure 10.10) (Takemoto et al., 2013). Note that the polymer–polymer conjugation efficiency between siRNA and the PAsp(DET) derivative using copper-free click chemistry (i.e., the reaction between azide and cyclooctyne) exceeded 95% by freeze-thawing of the reaction solution, most likely because of the enrichment effect of the solutes during freezing (freeze concentration), facilitating the collision of the reactants toward efficient reaction (Takemoto et al., 2012). The obtained siRNA conjugate and its polyplexes liberated monomeric siRNA at the late endosomal pH 5.0, whereas siRNA release was not observed at pH 7.4. In addition, siRNA conjugate-loaded polyplexes successfully induced the endosomal escape of siRNA payloads and gene silencing in cultured cancer cells.

5. OTHER STIMULI-RESPONSIVE NUCLEIC ACID DELIVERY

Apart from the redox potential and pH, there are various site-specific signals that can be used in bioresponsive nucleic acid delivery, e.g., tumor-related hypoxia, glucose, ATP, and enzymes. This section introduces enzyme and ATP-responsive formulations, which have been progressively developed for nucleic acid delivery.

5.1 Enzyme-Responsive Polymers

Enzymes, which consist of proteins, serve as catalysts for most biochemical reactions, including degradation and oxidization or reduction, under mild conditions, i.e., pH 5–8 and 37 °C (de la Rica, Aili, & Stevens, 2012; Ulijn, 2006; Zelzer et al., 2013). To date, thousands of enzymes have been identified in the human body and their site of action is highly specified (Ulijn, 2006). Thus, the introduction of an enzymatic substrate into polymers affords enzyme-responsive nucleic acid carriers that exert highly localized functions on demand. Among the numerous enzymatic substrates, enzymatically degradable peptide, nucleotide, and ester have been installed into polymer structures (Hu, Zhang, & Liu, 2012). Similar to the disulfide and acid-labile bonds, enzyme-responsive cleavable linkers can elicit site-specific structural change in the polymers and polyplexes for enhanced cellular internalization and the intracellular release of nucleic acids. For example, a substrate peptide for the lysosomal enzyme Cathepsin B was used in the polyplex disintegration and nucleic acid release within the lysosome (Figure 10.11(A)) (Chu, Johnson, & Pun, 2012). In this study, cationic oligomers comprising the Cathepsin B-substrate peptide were copolymerized with *N*-(2-hydroxypropyl)methacrylamide (HPMA), and the resulting polycation was mixed with pDNA for polyplex preparation.

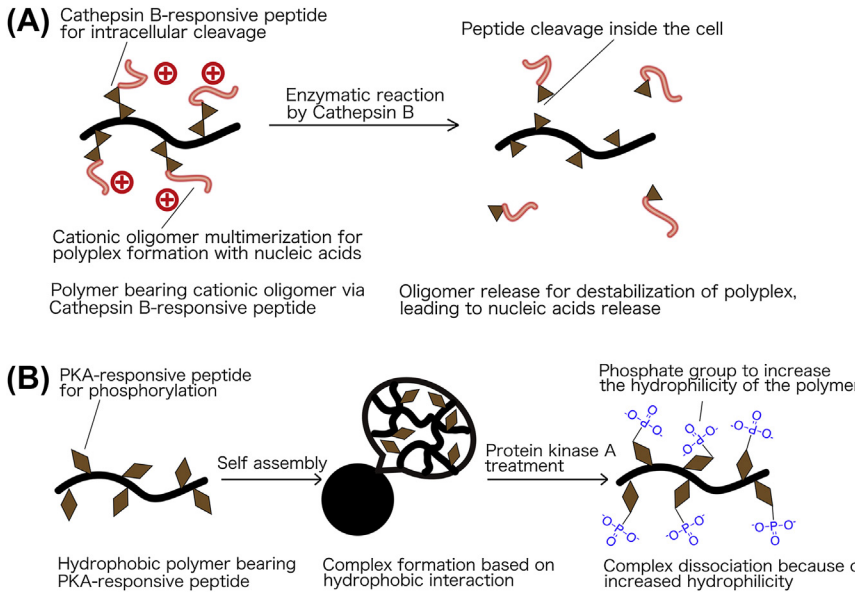


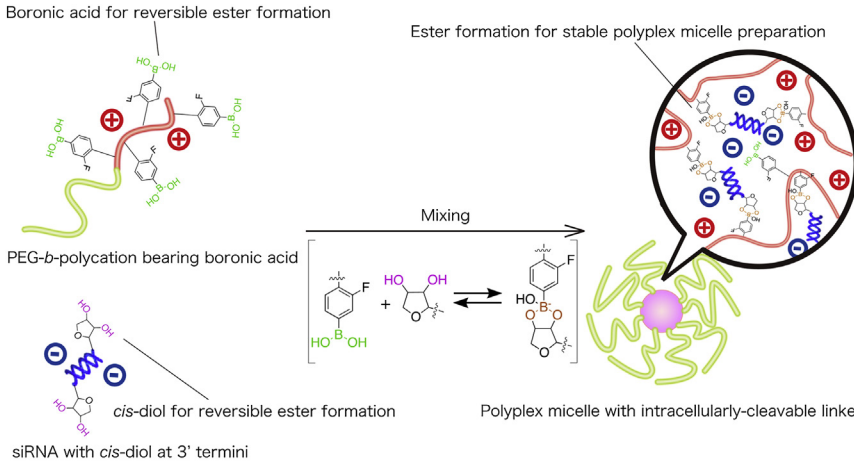
Figure 10.11 ***Polymer design in response to Cathepsin B (A) and protein kinase A (B).***

The obtained polyplex significantly facilitated the release of pDNA in the presence of Cathepsin B because of the cleavage of the cationic oligomers from the backbone polymer. Similarly, a substrate peptide for matrix metalloproteinase 2 (MMP-2), which is overexpressed in tumor tissues, was used to design carriers featuring tumor-specific responsibility (Hatakeyama et al., 2011; Yingyuad et al., 2013). The introduction of PEG on the carrier surface via the MMP2-substrate peptide was demonstrated to overcome the aforementioned PEG problem, i.e., the inefficient internalization of nucleic acid carriers by target cells, as well as nontarget cells. Thus, the PEG detachment triggered by tumor-specific enzymes can facilitate the interactions between carriers and cancer cell surface for enhanced tumor accumulation and cancer cell internalization. Notably, when systemically administered into subcutaneous fibrosarcoma tumor-bearing mice, the PEG-detachable siRNA carriers exhibited significantly enhanced tumor accumulation and greater gene silencing in the tumor tissue compared with the PEG-undetachable control without the MMP2-substrate peptide (Hatakeyama et al., 2011).

Nondegradable but structurally alterable peptides are also available for designing enzyme-responsive polymers. Hydrophobic polymers modified with a protein kinase A (PKA)-substrate peptide were reported to self-assemble in an aqueous solution because of hydrophobic interactions, whereas the assembly dissociated in the presence of PKA, because the phosphorylation of the peptide by PKA weakened the polymer hydrophobicity (Figure 10.11(B)) (Katayama, Sonoda, & Maeda, 2001; Sonoda et al., 2005). This strategy concurrently triggered the release of the payload from the assembly. Note that kinase- and phosphatase-based phosphorylation and dephosphorylation enable the reversible site-specific alteration of polymer hydrophilicity, directed toward ON/OFF switching of the payload release or the biological activity of the payload.

5.2 ATP-Responsive Polymers

ATP, which consists of adenosine and triphosphate, is the most abundant nucleotide in the body. The energy supplied by the hydrolysis of ATP to ADP (adenosine diphosphate) maintains various metabolisms, such as glycolysis, muscle contraction, and active transport. The ATP concentration is ~0.4 mM at the cell exterior and 1–10 mM at the cell interior (Gorman et al., 2007; Gribble et al., 2000; Traut, 1994). The design of ATP-responsive polymers for nucleic acid delivery is possible because of the large gap in the ATP concentration that is separated by the cellular membrane. In a previous



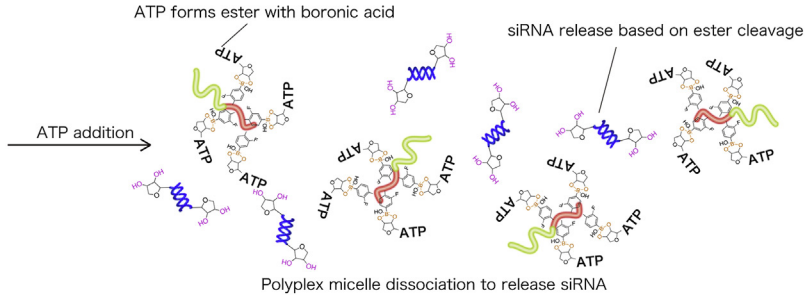


Figure 10.12 ***Strategy of ATP-responsive polyplex micelles.*** (See the color plate.)

study, an ATP-responsive siRNA polyplex micelle was constructed using phenylboronic acid (PBA) (Naito et al., 2012). A PBA moiety can form a reversible ester linkage with *cis*-diol molecules, e.g., ATP and the ribose at 3′ ends of siRNA (Figure 10.12) (Naito et al., 2012; Singh & Willson, 1999; Yan, Springsteen, Deeter, & Wang, 2004). Based on this underlying mechanism, siRNA was conjugated with PEG-*b*-PLys modified with PBA moieties in PLys side chains to form reversibly stabilized polyplex micelles. The formed micelles were demonstrated to selectively release siRNA payloads in response to high concentrations of ATP (>1 mM), whereas no siRNA release was observed at low concentrations of ATP (<0.6 mM). This result can be explained by the competitive interaction of ATP with the PBA moieties: thus, disturbing the binding between siRNA and PBA. The siRNA release could not be observed in the presence of 40 mM deoxythymidine monophosphate (dTMP) that is a nucleotide without *cis*-diol structure. This suggests that the

cis-diol structure in ATP is crucial for the cleavage of the reversible ester bond between the siRNA and PBA moieties. Finally, the siRNA-loaded micelles achieved higher gene silencing than the control micelles prepared with siRNA without *cis*-diol structure at the 3′ termini (Naito et al., 2012).

6. CONCLUSION AND FUTURE PERSPECTIVES

Polymeric materials enable the arbitrary design of nucleic acid carriers featuring programmed multifunctionality because of their potential modifiability. In particular, the installment of bioresponsive moieties into polymer structures enables the site-specific alteration of the carrier properties in response to various biological stimuli. Biological microenvironments are precisely regulated in each site (e.g., organs and organelles), generating their own stimuli. Using these stimuli, the bioresponsive carriers can fulfill the desired functionality, e.g., tumor-targeted accumulation and the intracellular release of nucleic acids. Nevertheless, to date, the stimuli used in the carrier design have been limited to a few biological environments. The diverse biological stimuli in the body will be further explored, supplying novel ideas for designing nucleic acid carriers. We believe that bioresponsive polymers can help tailored designs of nucleic acid carriers that realize successful nucleic acid therapeutics for treating incurable diseases.

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CHAPTER ELEVEN

Chitosan-Based Nanoparticles for Mucosal Delivery of RNAi Therapeutics

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Contents

Abstract

RNA interference (RNAi) gene silencing by small interfering RNAs (siRNAs) offers a potent and highly specific therapeutic strategy; however, enabling technologies that overcome extracellular and intracellular barriers are required. Polycation-based nanoparticles (termed polyplexes) composed of the polysaccharide chitosan have been used to facilitate delivery of siRNA across mucosal surfaces following local administration. This chapter describes the mucosal barriers that need to be addressed in order to design an effective mucosal delivery strategy and the utilization of the mucoadhesive properties of chitosan. Focus is given to

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preparation methods and the preclinical application of chitosan nanoparticles for respiratory and oral delivery of siRNA.

1. INTRODUCTION

RNA interference (RNAi) is an established tool for studying gene function (Agaard & Rossi, 2007; Paddison, Caudy, & Hannon, 2002) but also shows promise as an effective and specific therapeutic for the treatment of cancer (Miele et al., 2012; Takeshita & Ochiya, 2006), viral infections (Morrissey et al., 2005), and inflammatory diseases (Howard et al., 2009) through double-stranded small interfering RNA (siRNA)-mediated posttranscriptional gene silencing of disease-associated genes. Successful therapeutic applications of siRNA are dependent on efficient delivery to the target tissue and cells in order to facilitate knockdown of targeted transcripts that is compromised by the susceptibility of siRNA to nuclease degradation, rapid renal excretion, and insufficient entry into cells due to its polyanionic nature (Soutschek et al., 2004). siRNA incorporation in nanoscale particles can be used to overcome extracellular and intracellular barriers by installing protection, increased migration, extravasation, and endocytosis that promotes them as an enabling technology for the clinical realization of RNAi therapeutics (Howard, 2009).

Different routes of administration have been used for nanoparticle delivery of RNAi therapeutics (Howard, 2009) with intravenous injection as the primary method (Larson, Jackson, Chen, Rychahou, & Evers, 2007; Lewis, Hagstrom, Loomis, Wolff, & Herweijer, 2002). Elaborate designs containing stealth and targeted components, however, are required to overcome systemic barriers that include serum-induced aggregation, opsonization, capture by phagocytic components of the Mononuclear Phagocyte System (MPS), and nonspecific accumulation. Simplicity is the key to clinical translation; therefore, more simple designs and approaches are needed.

The mucosal route of administration is an attractive alternative to systemic delivery for RNAi therapeutics (Howard, 2009; Howard & Kjems, 2007). The mucosal surface that lines the respiratory, gastrointestinal, and genitourinary tracts is free from serum proteins; and the therapeutic can be applied directly at the site of a range of diseases common to the mucosal surface, e.g., influenza within the pulmonary epithelia (Tamura & Kurata, 2004) without the necessity to install stealth and targeting components. Moreover, a capability does exist to design systems able to overcome the mucosal barrier and enter the systemic circulation.

The mucosal lining presents a formidable biological barrier comprising of closely packed epithelial cells with an overlying mucus gel (Linden, Sutton, Karlsson, Korolik, & McGuckin, 2008). The application of bioadhesive materials to increase nanoparticle residence time and reduce clearance for effective delivery of RNAi therapeutics across these sites is an attractive strategy. The mucoadhesive (Soane et al., 1999) and mucopermeable (Artursson, Lindmark, Davis, & Illum, 1994) properties of chitosan promote its application for siRNA delivery across mucosal epithelium for mucosal disease treatment.

This chapter addresses the biological and physical barriers occurring at the mucosal surfaces that restrict uptake of luminal material and design considerations for chitosan-based siRNA delivery solutions. The RNAi pathways and design criteria for RNAi therapeutics relevant for efficiency increase and reduction of potential adverse effects will be discussed.

2. RNA INTERFERENCE

RNAi is a fundamental eukaryotic posttranscriptional silencing process involved in viral defense, limiting propagation of transposons and other mobile genetic elements, and cellular gene regulation facilitated by endogenous small noncoding microRNA (miRNA) and siRNA. Recent attention has been given to harnessing this process for therapeutic purposes by engagement of exogenous artificial RNAi triggers (or effector molecules) for downregulation of disease-related genes. The understanding of RNAi-mediated RNA processing have led to development of many different RNAi effector molecules employing either the miRNA or siRNA pathway at different entry points. Such effector molecules include double stranded RNA (dsRNA) such as 21–23 nt siRNA and Dicer substrates (DsiRNA), primary miRNA (pri-miRNA), or miRNA mimics and various siRNA designs that can be introduced directly or expressed from viral- or plasmid-driven vectors (Bramsen & Hansen, 2013; Jarlstad Olesen, Ballarín-González, & Howard, 2014).

2.1 The Endogenous Function of RNAi

Constitutive expression of the RNAi machinery emphasizes the importance of RNAi. Understanding the endogenous role of the RNAi machinery in the intrinsic normal growth of cells and development of organisms will lead to minimization of the adverse events when employing RNAi-based therapeutics. In fact, natural RNAi machinery not only keeps the

mobile transposable elements from disrupting the integrity of genomes (Aravin et al., 2001; Hamilton, Voinnet, Chappell, & Baulcombe, 2002; Tabara, Hill, Mello, Priess, & Kohara, 1999), but also participates in organism development (Carrington & Ambros, 2003; Grishok, Tabara, & Mello, 2000; Knight & Bass, 2001; Smardon et al., 2000), apoptosis (Brennecke, Hipfner, Stark, Russell, & Cohen, 2003; Carrington & Ambros, 2003), and embryonic development (Song & Wessel, 2007). Some of the components of the RNAi machinery have also been clearly established as effector proteins for the maturation of miRNAs. miRNAs have been found to be abundant in plants, flies, worms, and humans (Carrington & Ambros, 2003; Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001; Lee & Ambros, 2001; Llave, Kasschau, & Carrington, 2000), with more than 1800 miRNAs identified so far in *Homo sapiens* according to the miRBase database (http://www.mirbase.org). Several studies in cell lines and tissues suggest that miRNA expression is cell- or tissue-specific (Lee & Ambros, 2001; Llave et al., 2000); however, many miRNAs are uniformly expressed, suggesting their role in general gene regulation (Ruvkun, 2001). Thus, the regulatory mechanism offers cellular protection against parasitic nucleic acid sequences, carries out epigenetic as well as genetic alterations on one hand, and governs organisms' architecture and development on the other (Agrawal et al., 2003).

In general, the posttranscriptional silencing activity of RNAi is dependent on two pathways, namely the miRNA and siRNA pathways (Figure 11.1). These two major RNAi pathways will be described with the challenges and possible solutions for utilisation in RNAi-based therapeutics.

2.1.1 miRNA Pathway

The endogenous miRNA regulates cellular gene expression by annealing to the 3′ untranslated region (3′ UTR) of its target mRNA inducing either translational repression or cleavage. Initially, these are transcribed by RNA polymerase II as long primary miRNA (pri-miRNA), which are then cleaved into a 75–50 nt stem loop precursor miRNA (pre-miRNA) by a RNase III endoribonuclease, Drosha (Yi, Qin, Macara, & Cullen, 2003). Pre-miRNA is exported by Exportin-5 from the nucleus to the cytoplasm where it is further processed into 21–23 nt dsRNA with 2-nt 3′ overhangs by the RNase III endoribonuclease Dicer (Zamore, Tuschl, Sharp, & Bartel, 2000). The miRNA is then loaded into RNA-induced silencing complex (RISC) in which the passenger strand is removed and degraded allowing the remaining guide strand to either partially or extensively anneal to target miRNA in a sequence-specific manner, leading to translational repression or mRNA degradation, respectively.

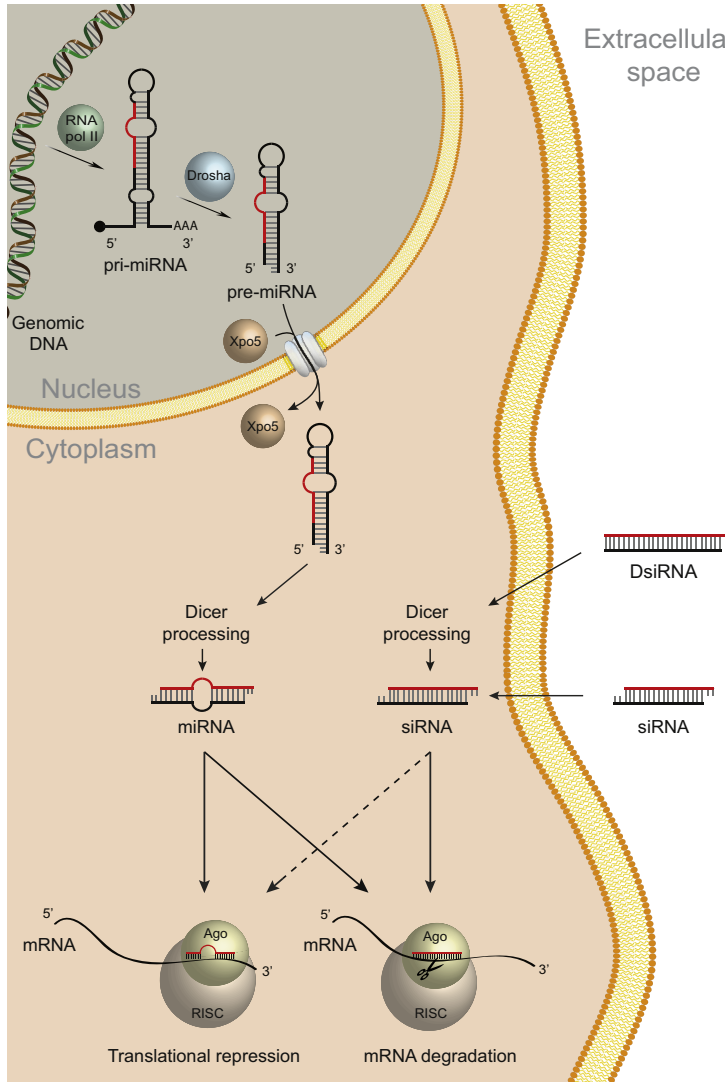

Figure 11.1 ***Posttranscriptional gene silencing through the microRNA (miRNA) and small interfering RNA (siRNA) pathways.*** Generation of mature miRNA requires the conversion of pri-RNA into pre-miRNA and subsequent cleavage into 21-nt-long duplex RNA (miRNA). These processing steps are mediated by the nuclear and cytoplasmic endoribonucleases known as Drosha and Dicer, respectively. Incorporation of mature miRNAs into RISC results in translational repression through partial complementarity between the guide strand and sequences within the 3′ UTR of the targeted mRNA. Additionally, miRNA can induce mRNA degradation through perfect guide strand–mRNA complementarity. Cleavage can also be mediated by siRNA molecules derived from Dicer processing of natural long dsRNA or synthetic siRNAs introduced into the cell as 21-nt-long duplex RNA or longer Dicer substrate RNA (DsiRNA). Dashed line indicates miRNA-like off-target activity of siRNAs effectors. 3′ UTR, 3′ untranslated region; Ago, Argonaute; DsiRNA, Dicer substrate siRNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; RNA pol II, RNA polymerase II; Xpo5, Exportin 5. *Taken from Jarlstad Olesen et al., 2014 with permission from Springer.*

2.1.2 siRNA Pathway

siRNA can be of natural or artificial origin, engaging the siRNA pathway either as longer 27-mer Dicer substrates (DsiRNA) that requires Dicer processing (Ghildiyal & Zamore, 2009; Kim et al., 2005; Rose et al., 2005) or shorter "matured" 21–23 nt dsRNA (siRNA) that are then incorporated into RISC. When bound to RISC, the guide strand targets mRNA by complementary base pairing resulting in enzymatic mRNA cleavage. In contrast to miRNA, siRNA directs mRNA cleavage through extensive rather than partial guide strand/mRNA complementarity. Synthetic siRNA permits incorporation of chemical modifications that provides more potent activity and reduction of nonspecific and off-target effects (Bramsen et al., 2010; Chen et al., 2008; Hamm et al., 2010; Jackson et al., 2006), while also having control over the dosage.

2.2 Clinical Utilization of RNAi Therapeutics

It has been shown that several siRNAs synthesized against different regions of the same target mRNA demonstrate different silencing efficiencies (Holen, Amarzguioui, Wiiger, Babaie, & Prydz, 2002). Optimal siRNA design, therefore, is crucial in order to obtain optimal silencing activity. However, despite extensive work analyzing the influence of parameters such as length, secondary structure, sugar backbone composition, and sequence specificity on silencing activity of siRNA, currently no consensus has been reached. Yet, some basic selection criteria for effective siRNA design have been suggested and summarized (Agrawal et al., 2003; Bramsen & Hansen, 2013).

In general, several requirements must be met before siRNA can be employed for therapeutic purposes: (1) siRNA is prone to enzymatic degradation and renal clearance and, therefore, requires protection; (2) siRNA has to enter its target cells to be functional without stimulating receptors of the innate immune systems; and (3) siRNA should gain access to its homologous region without inter alia epigenetic hindrances. As polyanionic siRNA molecules are not likely to cross the negatively charged cellular membrane unassisted, a major challenge for the application of siRNA therapy in the clinic is the design of a suitable carrier system that will protect the siRNA from degradation with limited or no immunostimulatory effects. Well-designed delivery systems can meet all the above-mentioned requirements and lead to effective disease treatment.

3. THE MUCOSAL BARRIER

Mucosal membranes of the respiratory, gastrointestinal, genitourinary tracts and eyes are exposed to the external environment and pathogens (Figure 11.2). Mucus is a viscoelastic gel comprised of hydrated proteins that

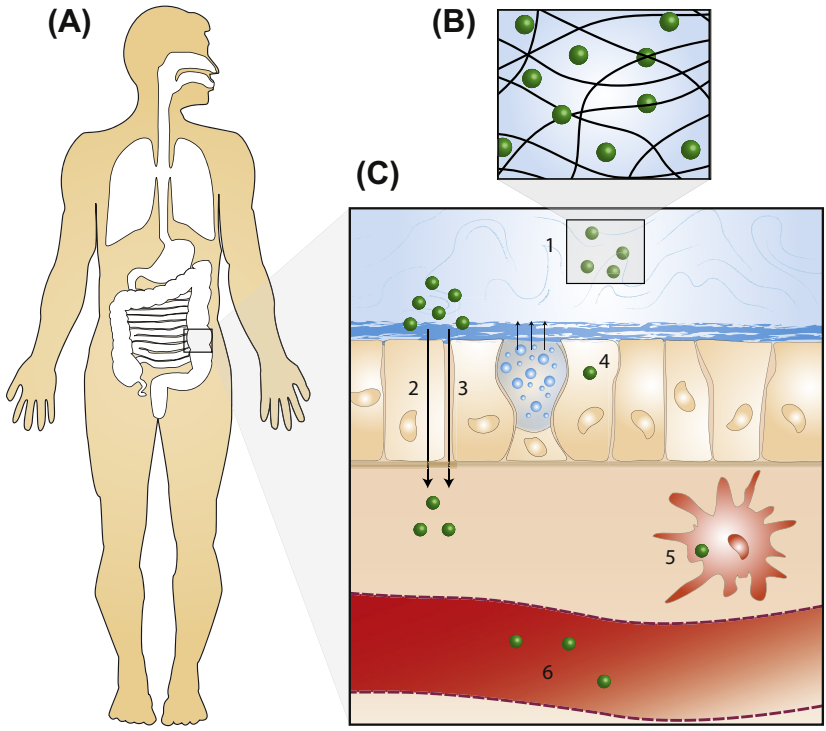

Figure 11.2 ***Schematic representation of nanoparticle uptake by mucosal routes.*** (A) Representation of the mucosal surfaces lining the respiratory, gastrointestinal, and genitourinary tracts. (B) Network of polymeric mucin fibers through which chitosan/small interfering RNA (siRNA) nanoparticles can penetrate. (C) Chitosan/siRNA nanoparticles are green (dark grey in print version) at the luminal surface encounter mucus are blue (light grey in print version) and epithelial barriers are beige (light grey in print version) before entering the mucosal tissue. When the first encountered mucus layer is breached the particles (1) penetrate the epithelial layer by transcytosis (2) or paracellular route. (3) Furthermore, endocytosis by epithelial- (4) and/or *lamina propria* cells (5) can occur, as well as the possibility of the nanoparticles gaining access to systemic translocation (6) by the portal vein or lymphatics of the gut.

overlay the mucosal membranes and functions as a protective barrier for mucosal tissue and, as such, compromises the delivery of RNAi therapeutics (Ballarín-González, et al., 2013a; Howard, 2009; Howard & Kjems, 2007). The primary role of mucus is to act as a first line of defense against intruding pathogens and toxins, while also facilitating continuous exchange of nutrients, water, and gases. Mucus is composed of ions, proteins, lipids, DNA, cellular debris, and approximately 0.5–40 MDa glycoproteins known as mucins—the main structural components secreted by goblet cells (Lai, Wang, Wirtz, & Hanes, 2009). Mucins form a heterogeneous network of polymeric fibers with pores of

~200–300 nm in diameter (Bansil, Celli, Hardcastle, & Turner, 2013). Other studies reveal a wide range of pore sizes depending on the methods used, from ~100 nm by using DMSO-mediated glutaraldehyde fixation and transmission electron microscopy (TEM), to 500–800 nm by using freeze substitution and TEM, and 1000–10,000 nm or larger by using various conventional electron microscopy procedures (Lai, Wang, Hida, Cone, & Hanes, 2010). The success of nanoparticle-based mucosal delivery is dependent on movement of particles through these pores; therefore, pore size and interaction with the mucin glycoproteins are important considerations in the design of mucosal-based nanoparticle systems.

Mucus assembles in two layers, an outer more viscous and mobile layer, and an underlying denser layer in contact with the epithelium (Johansson, Larsson, & Hansson, 2011). However, mucus variations such as thickness, rate of renewal, and pH are observed at different anatomical sites due to diverse tasks performed by the tissue and the particular luminal conditions. The mucus thickness in the human gastrointestinal tract ranges from approximately 50–450 μm in the stomach and 110–160 μm colon, while the thickness have been reported to be 30–40 μm in the eyes, 7–30 μm in the airway, and approximately 55 μm in the bronchiole. Furthermore, large differences in mucus renewal rates exist, from 20 min in the nasal cavity to 10–20 min in the respiratory tract, while every 4–6 h in the gastrointestinal tract has been shown in rats. This is an important consideration if utilizing mucoadhesive drug carriers to increase drug concentration and resident time at mucosal membranes, as a fast mucus renewal rate will compromise drug residence. pH differences occur at different sites, with mucus of the lungs and nasal cavity reported to be close to neutral, mucus of the eyes slightly basic, and stomach mucus has pH 1–2 at the luminal site, while pH 7 at the serosal side close to the underlying epithelial cells (Lai et al., 2009). All these features, however, may be altered during pathological states. Thickening of the mucus layer has been observed in the case of asthma, cystic fibrosis, and chronic obstructive pulmonary disease, while in ulcerative colitis, a decreased thickness of the mucus layer has been shown (Pullan et al., 1994). Parameters such as pH, mucus thickness, and renewal rate have to be considered in the mucosal drug delivery system design. For example, insufficient dissolution of the drug at a certain pH may inhibit drug release, while, low pH-degradable polymers can be used for triggered drug release in the stomach (Shang, Zhang, Chen, Liang, & Shi, 2013). Similarly, utilization of a system with slow mucus penetration into the pulmonary tract might be problematic due to a high mucus renewal in these regions.

After successful penetration into the mucus layer, siRNA has to overcome the epithelial cell barrier (Figure 11.2). In general, the mucosal epithelium is comprised of densely packed epithelial cells situated on a basement membrane, with site-specific differences in cell morphology and cell type composition. The epithelium of the small intestine comprises of simple columnar epithelium containing absorptive enterocytes displaying apical microvilli, mucin-secreting goblet cells, antimicrobial-secreting Paneth cells, and lymphoid-associated microfold or M cells (Howard, Thomas, Jenkins, Davis, & O'Hagan, 1994; Peterson & Artis, 2014), while the upper respiratory tract is composed of ciliated pseudo-stratified epithelium that facilitates removal of environmental and pathogenic particles (Rastogi, Ratner, & Prince, 2001). Differences in the epithelial cell barriers, therefore, need to be addressed for each particular delivery system. Materials that breach the mucus barrier may be translocated through the intestinal epithelium into the systemic circulation dependent on the physicochemical characteristics of material and the physiological state of the barrier (Martirosyan, Polet, Bazes, Sergent, & Schneider, 2012). Material can be taken across the epithelium by transcellular and/or paracellular routes. The main transcellular passage mechanism for nanoparticle transport across the epithelium is adsorptive or cell-mediated endocytosis. Modification of material properties or targeting specificities can be used to maximize delivery across the mucosa. It is generally accepted that the tight junctions restrict paracellular transport of micro-nanoparticles; however, mucopenetration enhancers, including chitosan, have been used to facilitate transient opening of the tight junctions and mediate paracellular movement of small molecules (Sonaje et al., 2012).

4. MUCOADHESIVE BIOMATERIALS

In recent years, much attention has been given to the design of particles, which associate with the mucus barrier and, hence, lower clearance rate (Ballarín-González et al., 2013b). Materials of choice have been the so-called mucoadhesive biomaterials. Mucoadhesion is the terminology used when a material, e.g., a polymer, is held together with mucus or a mucus membrane by interfacial forces (Hombach & Bernkop-Schnurch, 2010). Mucoadhesion is a complex process and numerous theories have been proposed to explain the mechanisms involved. There are six classical theories adapted from studies on the performance of several materials and polymer–polymer adhesion, they are the wetting theory, the

diffusion theory, the fracture theory, the electronic theory, the mechanical theory, and the adsorption theory (Carvalho, Bruschi, Evangelista, & Daflon Gremião, 2010; Harsulkar, Sreenivas, Mandade, & Wakade, 2011).

Both the mucus physiology and the structure of the polymer affect the mucoadhesive properties of the polymer and mucin. Mucin polymers are comprised of disulfide-linked monomers that contain repeated hydrophilic glycosylated amino acid sequences and hydrophobic cysteine-rich domains that can interact with particles by electrostatic, hydrogen and hydrophobic interactions. A crucial element for exploitation of mucoadhesion for mucosal drug delivery is the aforementioned bilayered structure of mucus, where the association of particles with the lower undisturbed layer will prevent clearance and enhance the bioavailability of the bioactive entity. Another positive effect from mucus association is increased viscosity due to greater cross-linking of mucus fibers, which in turn may lower the clearance rate (Lai et al., 2009). Mucoadhesion, therefore, could provide a solution to poor bioavailability of drugs with short residence at the absorption site. Improving the mucoadhesive ability of nanoparticles is, thus, an attractive approach to improve uptake of therapeutics at epithelial surfaces (Acosta, 2009; Behrens, Pena, Alonso, & Kissel, 2002; Chen, Sonaje, Chen, & Sung, 2011; Takeuchi, Yamamoto, & Kawashima, 2001; Yin et al., 2009).

Mucoadhesive biomaterials can be classified into four distinctive groups (Roy, Pal, Anis, Pramanik, & Prabhakar, 2009): (1) carboxylic-bearing hydrophilic polymers (e.g., carboxy-terminated polyvinyl pyrrolidine, carboxymethyl cellulose); (2) hydrophilic polymers that swell when in contact with water, e.g., hydrogels that adhere to the mucus membrane. These polymers can be further classified according to the charge of biomaterials, e.g., anionic polymers (carbopol, polyacrylates), cationic polymers (chitosan), and neutral/nonionic polymers (eudragit analogues); (3) thiolated polymers (e.g., thiolated (poly) acrylic acid and thiolated chitosan), where the presence of free thiol groups in the polymer chain mediates the formation of disulfide bonds with cysteine-rich subdomains present in mucin. Thiolation of polymers have been shown to enhance mucus interaction (Leitner, Walker, & Bernkop-Schnürch, 2003), and various thiolated chitosans have, thus, been synthesized (Bernkop-Schnürch, 2003; Schmitz, Grabovac, Palmberger, Hoffer, & Bernkop-Schnürch, 2008); and (4) lectin-based polymers that engage specifically to sugar/carbohydrate complexes attached to proteins and lipids (Bies, Lehr, & Woodley, 2004) with a capacity to bind with the intestinal and alveolar epithelium (Harsulkar et al., 2011).

One of the widely used mucoadhesive biopolymers is the polysaccharide chitosan (Soane, 1999), which has been utilized to form nanoparticles with siRNA and shown to exhibit mucosal gene silencing properties (Ballarín-González et al., 2013b; Howard et al., 2006; Liu et al., 2007; Nielsen et al., 2010). Mechanistic studies of interaction of chitosan and mucin have shown that the electrostatic interaction between the positively charged amines on the polymer chains and the negative sialic acid residues on the mucin glycoprotein plays a central role in the mucoadhesive properties (Bravo-Osuna, Vauthier, Farabollini, Palmieri, & Ponchel, 2007; He, Davis, & Illum, 1998; Salamat-Miller, Chittchang, & Johnston, 2005; Sogias, Williams, & Khutoryanskiy, 2008). More detailed studies were conducted by Sogias et al. (2008) in order to reveal the contribution of different physical interactions through manipulating chitosan structure by partial acetylation of the polymer. The authors demonstrated that mucoadhesive interactions between chitosan and mucin are complex and, that although the electrostatic attraction appears to be the major mechanism for chitosan mucoadhesion, there also seems to be contributions from hydrogen bonding and hydrophobic effects. These interactions can provide a prolonged contact between the chitosan-based nanoparticles and the absorptive surface, thereby, promoting their absorption capacity.

5. CHITOSAN

Chitosan is a deacetylated derivative of chitin, which is the second most abundant natural polymer. It is found in the exoskeleton of insects, crustacean shells, and fungi (Kumar, 2000). The use of chitosan in pharmaceutical formulations is a relatively new development but has been used for proteins (Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001), peptides (Ma, Lim, & Lim, 2005), DNA (Mansouri et al., 2004; Mao et al., 2001), and recently siRNA (Ballarin-Gonzalez & Howard, 2012; Howard et al., 2006, 2009; Liu et al., 2007). In addition to its wide availability, it is nontoxic, biocompatible, and biodegradable. It is used as a dietary supplement for preventing fat absorption and is recognized as GRAS (Generally Recognized As Safe) by the FDA (Garcia-Fuentes & Alonso, 2012).

Chitosan consists of repeating units of β-1,4 linked *N*-acetyl-D-glucosamine and D-glucosamine whose ratio determines the degree of deacetylation (DD) of the polymer. The DD has a significant effect on the solubility and rheological properties of the polymer, including the ability to interact electrostatically with polyanions such as siRNA. The amine group

has a pKa in the range of 5.5–6.5 that ensures chitosan is insoluble at neutral pH, but soluble and positively charged at acidic pH. Thus, positively charged chitosan interaction and complex stability with polyanionic species such as siRNA at low pH promote its application for siRNA drug delivery.

The glucosamine component provides amine groups that facilitate the mucoadhesiveness, mucopermeable properties of chitosan, and complex formation with polyanions. This complexation provides protection against acidic denaturation and enzymatic degradation and the capability to prolong the residence time of nanoparticles in the small intestine (Sakloetsakun, Perera, Hombach, Millotti, & Bernkop-Schnürch, 2010; Sonia & Sharma, 2011). Evidence for the mucoadhesive properties of chitosan delivery systems is provided by the reduced clearance rate of chitosan from the mucosal surface, e.g., nasal cavity, thereby, increasing the contact time of the delivery system with the mucosa, providing potential to increase the bioavailability of drugs incorporated into these systems (Soane et al., 1999). Moreover, chitosan can mediate the opening of tight junctions between epithelial cells reversibly, thus, facilitating the paracellular transport of hydrophilic macromolecules (Amidi, Mastrobattista, Jiskoot, & Hennink, 2010; Sonaje et al., 2011, 2012). Many studies, therefore, have focused on the use of chitosan-based nanoparticles to improve the oral bioavailability of macromolecular agents (Chen, Wong et al., 2009; Nguyen et al., 2011; Sarmento, Ribeiro, Veiga, Ferreira, & Neufeld, 2007). In addition, recent studies suggest that inhaled chitosan/siRNA formulations may offer a fast, potent, and easily administrable therapy against respiratory diseases in humans (Nielsen et al., 2010; Sharma, Somavarapu, Colombani, Govind, & Taylor, 2013). Studies have shown that the mucoadhesive feature of chitosan and its ability to mediate the opening of tight junctions depend strongly on its protonation degree (Bravo-Osuna et al., 2007; Sadeghi et al., 2008; Sandri et al., 2010).

6. CHITOSAN/SIRNA NANOPARTICLES

A promising class of nanoparticles based on self-assembly of siRNA with polycations is polycationic particles, also termed polyplexes (Howard, 2009; Howard & Kjems, 2007; Howard et al., 2009). A cationic cyclodextrin-bearing polymer forming particles with siRNA has been taken into clinical trials (Davis, 2009; Davis et al., 2010). Submicron particles (approximately 50–300 nm) are formed by entropy-driven self-assembly between amine-bearing cationic polymers and the phosphate-bearing anionic siRNA to form a polyelectrolyte complex. Due to the glucosamine subunit

in chitosan, in which the pKa of ammonium cations is 6.5, chitosan will be cationic at pH 6.5 or below and complex with siRNA.

Several properties of chitosan need to be taken into account and optimized in order to achieve efficient gene silencing with chitosan-based siRNA delivery systems. These include molecular weight (MW) of chitosan, DD, the N:P ratio (ratio of polymer bearing amines to phosphates in the siRNA backbone) as well as the particle production method. The N:P, MW and DD of chitosan determine the physicochemical properties, such as size and stability, and consequent biological activity. In vitro studies have indicated that high N:P ratio (>20) polyplexes are optimal for cellular internalization due to a combination of discrete particle formation (Liu et al., 2007) and the presence of excess polycation (Liu et al., 2007; Thibault et al., 2011), which has proved effective for in vivo gene silencing (Howard et al., 2009; Nielsen et al., 2010). DD or charge density has been shown to highly influence the ability to complex with polyanions, e.g., siRNA, where a high DD favors the formation of efficient nanoparticles (Liu et al., 2007; Mao, Sun, & Kissel, 2010). Recent studies have demonstrated that the mucoadhesive feature of chitosan and its ability to mediate the opening of tight junctions depend strongly on its protonation degree (Bravo-Osuna et al., 2007).

The particle size of the chitosan/siRNA complex is dependent on chitosan MW. Katas et al. (2006) showed that a smaller mean particle size of chitosan nanoparticles was obtained when the lower MW of chitosan (110 kDa) was used compared to the higher MW chitosan (270 kDa). Liu et al. (2007) indicated that the chitosan molecules (MW 64.8–170 kDa), 5–10 times the length of the siRNA (MW 13.36 kDa), could form stable complexes with siRNA through electrostatic forces along the same or adjacent chitosan molecules. In contrast, chitosan with a low MW (10 kDa) could not complex and compact siRNA into stable particles, resulting in the formation of large aggregates and almost no knockdown (Liu et al., 2007). In this paper, the authors commented that high charge was necessary for chitosan/siRNA nanoparticle stability in contrast to chitosan/DNA nanoparticles (Sato, Ishii, & Okahata, 2001), indicating the stronger interactive forces of large polyanionic species in comparison to siRNA.

Chitosan offers enormous structural and functional versatility. It has been demonstrated that tailoring of the degree of polymerization, the fraction of *N*-acetylated units, and the chain architecture is essential for optimizing the delivery efficiency of the nanoparticles (Malmo, Vårum, & Strand,

2011; Strand et al., 2009; Strand, Issa, Christensen, Vårum, & Artursson, 2008). Chitosan-based gene carriers could neutralize the negative charge of nucleic acids and demonstrate significant versatility in terms of rigidity, hydrophobicity/hydrophilicity, charge density, biodegradability, and MW. All these features can be adjusted in order to achieve an optimal complexation with siRNA.

6.1 Production of Chitosan/siRNA Nanoparticles

Chitosan/siRNA nanoparticles can be produced by simple electrostatic interactions. In our laboratory, we have developed a self-assembly process, without the requirement of a cross-linker using both the bulk and microdroplet methods (Figure 11.3(A) and (B), respectively).

6.1.1 Bulk Formulation

Chitosan is first dissolved in sodium acetate buffer (300 mM, pH 5.5) and an appropriate amount of siRNA, to obtain the desired N:P ratio, is then added to the solution by slow continual motion while stirring, and the mixture was left to stir for an hour at room temperature. Nanoparticles are formed by electrostatic self-assembly of the component parts (Andersen, Howard, & Kjems, 2009). It was shown that the physicochemical characteristics of these nanoparticles in a size range of 40–600 nm are strongly dependent on N:P ratio; with a tendency for smaller particle size at higher N:P (Liu et al., 2007).

6.1.2 Microdroplet Formulation

The microdroplet-based synthesis of nucleic acids loaded polyplexes is a novel tool aimed at better control of physical properties of nanocomplexes, e.g., size, shape, and stability (Ho, Grigsby, Zhao, & Leong, 2011). The microdroplet formulation method developed at Aarhus University for chitosan/siRNA nanoparticles is based on bulk formulation with the extra addition of an oil phase and consequent microdroplet reaction incubators. This results in a stratified appearance of the components with siRNA in the top layer, chitosan in the intermediate layer, and oil in the bottom layer. Microdroplets with chitosan/siRNA polyplexes form in the top phase after vortexing and incubation for ~10 min. The droplets are consequently disrupted by adding matched drop release reagent (Ho et al., 2011). Finally, the particle solution can be harvested from the upper aqueous phase.

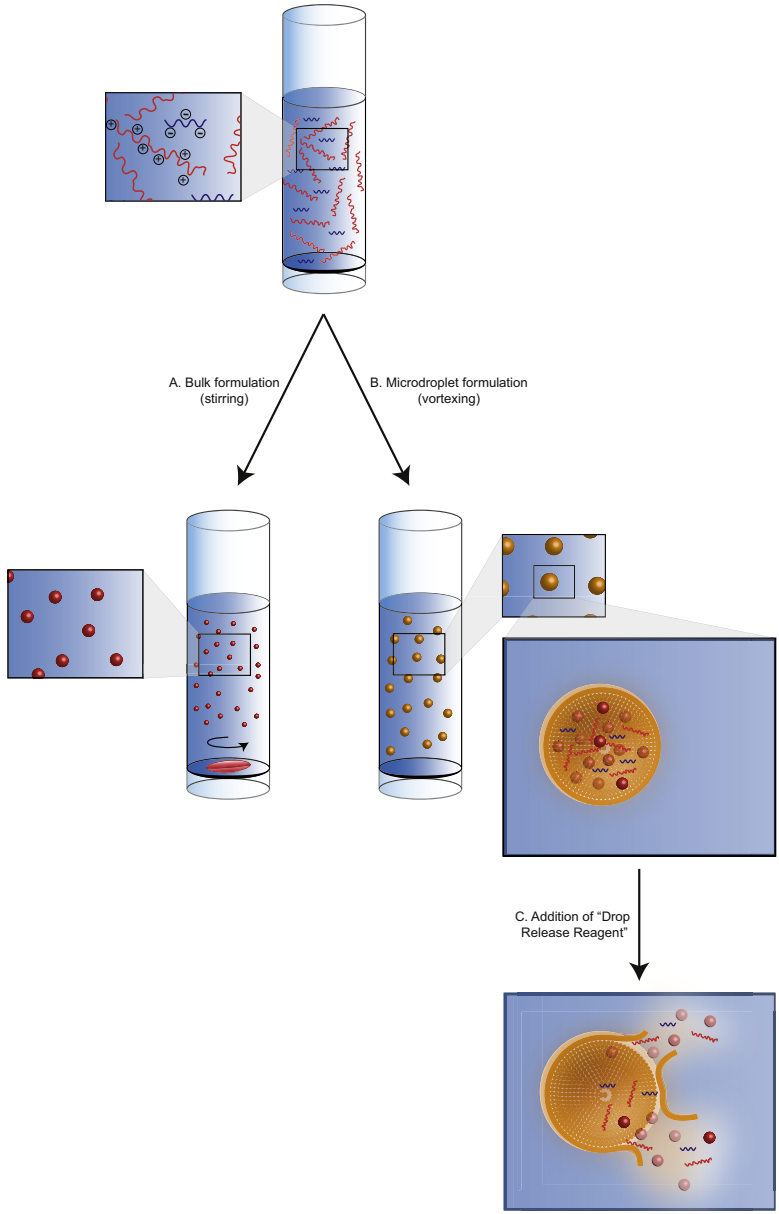

Figure 11.3 ***Preparation of chitosan/small interfering RNA (siRNA) nanoparticles.*** Chitosan/siRNA nanoparticles (termed polyplexes) prepared by a (A) bulk or (B) microdroplet approach. At pH below 6.5, chitosan and siRNA will spontaneously form polyplexes through electrostatic interactions when mixed under stirring (A) or vortexing (B). Microdroplet formulation will restrict chitosan and siRNA association in a more defined area. Formed microdroplets can then be disrupted using "Drop Release Reagent" (C) and the chitosan/siRNA polyplexes consequently harvested.

7. CHITOSAN/SIRNA NANOPARTICLES FOR DELIVERY ACROSS MUCOSAL BARRIERS

As mentioned earlier, mucosal delivery represents an attractive alternative to systemic administration, however, it poses a barrier to RNAi-based therapeutics both in naked and nanoparticle forms. This section will describe studies in which chitosan/siRNA delivery systems have been used for mucosal applications.

7.1 Respiratory Delivery

Delivery of siRNA to the lungs, whether naked or incorporated in a particle, needs to address the branching of airways and the mucus layer covering the conducting segments. Ciliated cells are abundant in the nasal cavity and trachea, with apical cilia working in coordinated sweeps to transport mucus along with trapped material toward the esophagus. The respiratory system also provides an opportunity for drugs to reach the systemic circulation by uptake across the thin epithelium of the alveoli.

Chitosan-based nanoparticle delivery of siRNA was first introduced by Howard and coworkers, who demonstrated gene silencing in vitro and in vivo (Howard et al., 2006). Our group investigated the nasal route for the administration of chitosan/siRNA nanoparticles to mediate RNAi at respiratory sites (Howard et al., 2006). A green fluorescent protein (GFP) silencing ~40% within bronchiole epithelial cells in a transgenic GFP mice model was obtained when the mice were exposed to 30 μg of siRNA per dose over five consecutive days of the chitosan/siRNA polyplexes at various N:P ratios. Parameters such as high MW (~100 kDa) and high DD (>80%) of chitosan at N:P > 30 showed improved polyplex formation, stability, and knockdown in vitro (Liu et al., 2007). However, intranasal administration may lead to nanoparticle loss during the nasal-to-lung transit as a consequence of adherence in the nasal cavity. Chitosan/siRNA aerosols were developed and investigated to improve pulmonary delivery of siRNA. In a more recent study by our group, the airway deposition of the chitosan/siRNA particle system was improved with an aerosolized formulation using a nebulizing catheter. The GFP silencing was evaluated in vivo after intratracheal administration via the nebulizing catheter in a GFP transgenic mouse model and was compared to the intranasal route (Nielsen et al., 2010). The siRNA distribution in the lungs was improved with the intratracheal route, and 37% GFP inhibition was obtained in the alveolar

and bronchiolar regions. The gene silencing results were similar to those obtained with intranasal administration (Howard et al., 2006), however, lower siRNA doses were used via the intratracheal route (0.26 μg siRNA per dose) compared with the intranasal route (30 μg). The low dose is a significant step toward reduction of possible dose-associated off-target and immunological side effects associated with siRNA (Robbins et al., 2008). The results obtained show that the developed aerosolized chitosan/siRNA nanoparticle system is effective and demonstrates good ability to mediate RNAi at respiratory sites for improved pulmonary siRNA delivery and gene silencing, and constitutes a promising approach for the treatment of pulmonary diseases.

7.2 Oral Delivery

Oral delivery is an attractive route for drug administration and is considered to be the most favorable in terms of cost-effectiveness, ease of administration, and patient compliance. This route potentially provides rapid systemic distribution of the drug due to the high adsorptive surface area of the gastrointestinal tract (Hillery, Lloyd, & Swarbrick, 2002). Oral administration of RNAi-based drugs offers great potential for both the treatment of diseases occurring locally within the gastrointestinal tract, such as inflammatory bowel diseases, and to combat systemic pathologic conditions. There are, however, considerable obstacles associated with this route of administration. Exposure to a highly active enzymatic environment in the gastrointestinal tract, extreme pH conditions, and the existence of a mucosal epithelial barrier are the main challenges for oral delivery of RNAi therapeutics.

Once the harsh conditions of the gastrointestinal tract are overcome, the uptake of a therapeutic compound via the oral route will depend upon the interactions of the drugs with the mucus components as well as the thickness of this layer, which in experimental animal models has been shown to vary along the gastrointestinal tract (Atuma, Strugala, Allen, & Holm, 2001; Varum, Veiga, Sousa, & Basit, 2010). It is unlikely that naked siRNA can overcome all the gastrointestinal barriers and is prone to gastric degradation. However, binding of specific ligands may facilitate uptake demonstrated in different cell types (Kumar et al., 2007; Nishina et al., 2008; Soutschek et al., 2004; Wolfrum et al., 2007). The pros and cons associated with the siRNA delivery via the oral route are summarized in a recent review paper (Ballarín-González et al., 2013a).

The feasibility of a simple chitosan-based polyplex system to deliver siRNA after oral administration was recently investigated by our group in

mice after oral gavage (Ballarín-González et al., 2013b). We showed that siRNA was efficiently protected by the chitosan, resulting in a deposition throughout the gastrointestinal tract and accumulation in systemic organs, e.g., liver, spleen, and kidney in an N:P ratio-dependent manner at 1 h after oral gavage. This work suggests that this system could provide an oral delivery platform possessing a potential to treat local and systemic diseases by siRNA. We are currently working on demonstrating functional gene silencing effects using this oral delivery platform.

8. CONSIDERATIONS AND FUTURE PERSPECTIVES

The potency and selectivity of RNAi make it a highly attractive therapeutic modality. Site-specific delivery, however, remains one of the key challenges that needs to be overcome in order to realize its clinical potential. Enabling technologies such as nanoparticles have been developed for overcoming extracellular and intracellular delivery requirements, where the route of administration and barrier encountered determine the nanoparticle design. Barriers associated with systemic administration by intravenous injection include serum-induced aggregation, first-pass hepatic clearance, protein opsonization, and MPS capture, and thus, require stealth and targeting technologies to be built into the nanocarrier design. The mucosal route is an attractive alternative to intravenous injection due to direct delivery at the site of many pathological conditions without the necessity for elaborate nanocarrier designs. Local delivery at mucosal surfaces offers the advantages of being readily accessible and enables delivery of nanoparticles directly to the target site to achieve maximum efficacy and minimum side effects at lower doses (Howard, 2009; Howard & Kjems, 2007; Peer & Lieberman, 2011). Hence, the development of systems for drug and gene delivery to mucosal surfaces (e.g., lung airways, gastrointestinal tract, female reproductive tract, nose, eyes) is of high interest and shows promise for delivery of siRNA that could lead to rapid clinical translation of mucosal-based RNAi therapeutics.

Polycationic nanoparticles formed by complexation between cationic polymers and siRNA, while intrinsically unstable in serum and susceptible to phagocytic capture, are ideally suited for mucosal delivery in a serum-free environment. The mucoadhesive property of chitosan and natural origin promotes its inclusion in mucosal delivery systems. The transfection efficiency of chitosan/siRNA nanoparticles depends on a series of physical and

formulation parameters, such as MW, DD, chitosan modifications, and N:P ratio. Data available so far indicate that complexes formed with a low MW and low DD chitosan would not be sufficiently stable to transfect cells efficiently. On the other hand, complexes formed with a high MW and high DD would be highly stable and may lead to low or delayed transfection. In addition, free chitosan at high N:P ratios is likely to facilitate greater mucus interaction and improves transit across mucosal surfaces. Moreover, the chitosan features could be tuned or given new ones via chemical modifications of the polymer (Park, Saravanakumar, Kim, & Kwon, 2010). Polycations (Hong et al., 2006; Mecke et al., 2005) and polyplexes (Chen, Hessler et al., 2009) may exhibit cell plasma membrane damage dependent on, for example, polymer charge density (Hong et al., 2006). Different approaches including chemical modifications, however, are available to overcome the potential toxicity of polycations (Ballarín-González & Howard, 2012).

Recently it has been shown that nanoparticles coated with low MW poly(ethylene glycol) (PEG) possess hydrophilic and near-neutrally charged surfaces that minimize mucoadhesion by reducing hydrophobic or electrostatic interactions (Lai et al., 2007). Dense coatings of PEG allow nanoparticles to rapidly penetrate through highly viscoelastic human mucus secretions by minimizing adhesive interactions between nanoparticles and mucus (Lai et al., 2007; Lai, Suk et al., 2011; Wang et al., 2008). It was shown that particles with a neutral surface charge had a greater diffusion rate compared to their negatively or positively charged counterpart. In this case, not the charge but the core hydrophobicity dominates in particle–mucin interaction (Lai, Wang, & Hanes, 2009). Thus, the development of mucoinert surfaces is another approach to achieve efficient drug delivery at mucosal surfaces that involves a fine balancing of interactions between particles and mucus.

The development of formulations capable of controlled release of drug at the mucosa particularly for particles residing within the mucus is an attractive property. Recently, a poly(lactic-co-glycolic acid) (PLGA) encapsulated chitosan/siRNA nanoparticle system was reported by Chen et al. (2012), where an improved release profile from the nanoparticle system was shown with a weak alkaline pretreatment. In the same alkaline pretreatment conditions, the PLGA-chitosan/siRNA nanofibers showed about 50% increase of the in vitro knockdown efficacy of EGFP.

Multivalent interactions between particles and the mucus network are the main determinants of particle diffusion. The particle surface charge, density,

and pH of mucin hydrogels were shown to alter particle diffusion rate in the mucus (Lieleg, Vladescu, & Ribbeck, 2010). Electrostatic interaction between the positively charged chitosan and the negatively charged binding sites of the mucin fibers, e.g., sialic groups, may induce particle dissociation due to the competitive forces of the mucin for the chitosan (De Campos, Sanchez, Gref, Calvo, & Alonso, 2003; Gaserod, Jolliffe, Hampson, Dettmar, & Skjak-Braek, 1998; Illum, Jabbal-Gill, Hinchcliffe, Fisher, & Davis, 2001). Recent work by our group, using a fluorescence-activated reporter system and microfluidic mucin barrier in vitro model indicated chitosan/siRNA polyplex disassembly when exposed to mucins (Thomsen et al., 2014). Results show that upon 30-min incubation of chitosan/siRNA nanoparticles with porcine gastric mucins, a decomplexation of nanocarriers occurs, where the decomplexation increases with increased N:P ratio. Moreover, the siRNA release from the chitosan/siRNA complexes remained constant within 30 min and the maximal disassembly correlates with the highest mucin concentration. Thus, a nanocarrier disassembly and subsequent triggered release and transit of siRNA upon interaction with mucins could be achieved that promotes the use of chemically modified RNAi therapeutics to enter the cells unassisted after release.

In addition to the necessity to select an ideal carrier system, the effective therapeutic application of siRNA-based drugs requires optimal siRNA designs that reduce nonspecific immune stimulation and maximize potency and delivery solutions. The design of the RNAi therapeutics is, therefore, an important consideration to reduce off-target effects, nonspecific immune stimulation and maximize potency. Unintentional targeting of genes in a manner resembling miRNAs activity or through the selection during RISC activation of the "wrong" guide strand should be avoided. To achieve this, different strategies based on chemical modification and siRNA strand design can be followed to minimize these "off-target" effects (Jarlstad Olesen et al., 2014; Bramsen & Hansen, 2013). Chemical modifications of siRNA can increase the stability of the duplex as well as help to overcome some of the siRNA side effects, such as the activation of an immune response (Kanasty, Whitehead, Vegas, & Anderson, 2012).

It is worth noting that the mucosal administration route offers an opportunity for transmucosal transfer of drugs into the systemic circulation. Recent work from our group showed N:P dependent accumulation of chitosan/siRNA nanoparticles in systemic tissue after oral delivery (Ballarín-González, et al., 2013b).

As an alternative to intravenous injection for treatment of systemic diseases without the necessity for stealth or targeting, we have developed an

intraperitoneal therapeutic strategy based on phagocytic capture of chitosan/siRNA particles (Howard et al., 2009; Nawroth et al., 2013). During inflamed conditions, macrophages can be recruited to the inflammatory site (Kinne, Brauer, Stuhlmuller, Palombo-Kinne, & Burmester, 2000; Simon et al., 2001), where an effective loading of chitosan nanoparticles as a consequence of the predisposition of particulates for phagocytic capture and the specific interaction of chitosan with mannose receptors can be utilized for silencing disease-relevant targets such as tumor necrosis factor. Injection in a serum-free, macrophage-rich environment such as the intraperitoneal cavity and consequent macrophage migration and recruitment to distal sites of inflammation can be used to transport nanoparticles without detection by the host immune system. Fluorescent-labeled siRNA containing chitosan nanoparticles was shown to enter peritoneal macrophages prominent in the development and progression of arthritis as a method to overcome the problem of nanoparticle instability and clearance associated with intravenous administration (Howard et al., 2009). Furthermore, the approach exploits the susceptibility of nanoparticles for phagocytic capture by MPS cells (Howard et al., 2009), enhanced by carbohydrate recognition by peritoneal macrophages, demonstrated also for chitosan-coated nanoparticles administered intraperitoneally (Ikehara et al., 2006), as well as the ability of this nanoparticle system to elicit gene silencing in "difficult-to-silence" primary macrophages (Howard et al., 2006). This "Trojan horse" approach offers an attractive alternative to systemic injection of nanoparticles for the treatment of diseases such as rheumatoid arthritis, psoriasis, systemic lupus erythematosus, inflammatory bowel diseases, and many types of cancers.

The application of chitosan/siRNA polyplexes for mucosal delivery of siRNA offers a highly promising approach with design characteristics tailored for mucosal transport with preclinical evidence supporting the further development as an enabling technology for mucosal siRNA drugs.

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CHAPTER TWELVE

Polycation-Mediated Integrated Cell Death Processes

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Abstract

One of the major challenges in the field of nucleic acid delivery is the design of delivery vehicles with attributes that render them safe as well as efficient in transfection. To this end, polycationic vectors have been intensely investigated with native polyethylenimines (PEIs) being the gold standard. PEIs are highly efficient transfectants, but depending on their architecture and size they induce cytotoxicity through different modes of cell death pathways. Here, we briefly review dynamic and integrated cell death processes and pathways, and discuss considerations in cell death assay design and their interpretation in relation to PEIs and PEI-based engineered vectors, which are also translatable for the design and studying the safety of other transfectants.

1. INTRODUCTION: CELL DEATH DEFINITIONS, GUIDELINES, AND PROCESSES

Attempts to create cell death guidelines are being made in the cell death community (Galluzzi et al., 2007, 2009; Klionsky et al., 2012; Kroemer et al., 2009) and are important to employ in all research disciplines investigating cytotoxicity and cell death in order to avoid confusion, accelerate the research progress, and bridge the gaps in experimental therapeutics. This section will thus attempt to introduce various cell death nomenclature, mechanisms and the interplay between them in accordance to the guidelines of the Nomenclature Committee on Cell Death (NCCD) (Galluzzi et al., 2007, 2009; Kroemer et al., 2009).

In 2005, the editors of Cell Death and Differentiation created NCCD that suggested that a clear separation between dead cells as opposed to dying cells should be made based on morphological features. A cell is declared dead when: (1) its plasma membrane integrity is lost as evident by incorporation of vital dyes (in vitro); (2) complete cell and nuclear fragmentation has occurred (frequently referred to as apoptotic bodies); and/or (3) adjacent cells have engulfed the fragments or corpse of dead cells (in vivo) (Kroemer et al., 2005). The most extensively studied cell death mechanism to date is undoubtedly apoptosis. The term apoptosis was coined by Kerr, Wyllie, and Currie (1972) defining specific morphological features such as rounding up of cells, pyknosis (nuclear condensation), karyorrhexis (nuclear fragmentation), minor changes of cytoplasmic organelles, plasma membrane blebbing (Figure 12.1), and engulfment by phagocytes (in vivo) (Kerr et al., 1972; Kroemer et al., 2009).

With regards to the processes and characteristics that have been correlated to apoptosis, such as it being a programmed cell death or occurring with caspase activation, nuclear fragmentation, and being classified

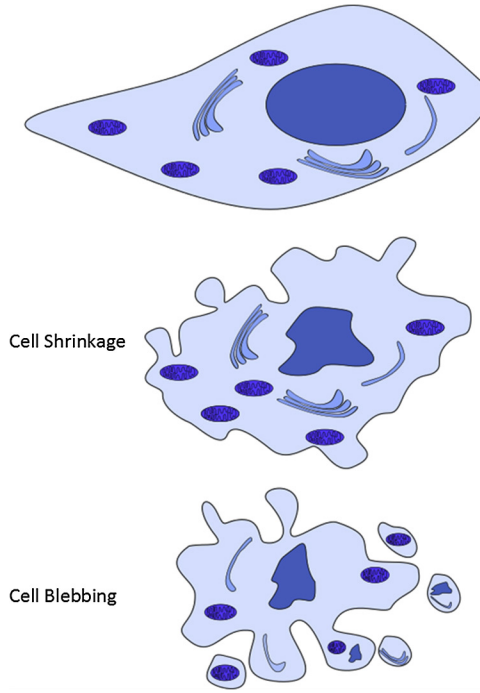

Figure 12.1 ***Schematic representation of an apoptotic cell.*** Apoptosis is characterized by cellular shrinking followed by nuclear condensation/fragmentation and membrane blebbing (e.g., cytoplasmic and endoplasmic reticulum (ER) origin). Plasma membrane damage occurs as a late event in apoptotic cells.

as a nonimmunogenic death, the NCCD stress that it is important to bear in mind that apoptosis can occur independent of caspases, and caspase activation can occur without occurrence of cell death (Galluzzi, Joza, et al., 2008; Galluzzi, Vicencio, et al., 2008). Similarly, an apoptotic cell death can occur without oligonucleosomal fragmentation (karyorrhexis) and despite earlier notions classifying necrosis as accidental cell death, numerous investigations have demonstrated that programmed necrosis can occur. Furthermore, it has been demonstrated that there are situations where cell death exhibiting apoptotic features can be immunogenic, while necrotic cells do not always have to induce an immunogenic response (Casares et al., 2005; Obeid et al., 2007; Zitvogel et al., 2004). In addition, similar to apoptosis and programmed necrosis, autophagy is also a programmed cell death. To this end, noninflammatory or nonimmunogenic death, programmed cell death, nuclear fragmentation, or caspase activation should not be used as synonyms or definite hallmarks of apoptosis (Kroemer et al., 2009). Rather, a detailed investigation

of morphological features together with biochemical/enzymatic and, if possible, immunological studies should be performed to delineate the mode of cell death as precisely as possible. This will help to eliminate oversimplified or even inaccurate interpretation of data and instead aid to gather information in a standardized and accurate manner (Galluzzi et al., 2007). In relation to interpretation of data, a standardized nomenclature when presenting data is also essential. As mentioned above, one should discriminate between the process of dying cells and dead cells as an end point. Dying cells can undergo several different processes and the presence or absence of these mechanisms should not be defining the mode of cell death as apoptotic or necrotic, but rather describing the path leading to the specific type of cell death. In accordance with this, cell death could be described as for example; "caspase-dependent/independent apoptosis" (Galluzzi et al., 2007). Terminology such as "percentage of apoptosis" should be avoided and terminology describing the actual readout such as "percentage propium iodide or AnnexinV positive/negative cells" should be used (Kroemer et al., 2009). It is also essential to address the interplay between the different modes of cell death pathways and even within a particular death pathway. For instance, heterogeneity in the triggering and execution of different biochemical pathways occurs in apoptosis, but they all generate the same morphological features (Danial & Korsmeyer, 2004; Kroemer et al., 2009).

1.1 Apoptosis (Type 1 Cell Death)

Since 1972, when the term apoptosis was first coined (Kerr et al., 1972), this mode of cell death has been shown to be a highly regulated cascade of events that plays an essential role in homeostasis. Indeed, deregulation of apoptosis has proven to have severe consequences. In homeostasis of renewing tissues, apoptosis helps to balance the proliferating cells and helps to eliminate damaged cells as well as playing a role in pathogen clearance. It has also been shown to be an essential part of embryogenesis and development of organs (Strasser, O'Connor, & Dixit, 2000; Strasser, Cory, & Adams, 2011; Trambas & Griffiths, 2003). Several different pathways have been demonstrated to lead to an apoptotic mode of cell death and over the years, major regulators such as the Bcl-2 family whose genes were discovered in the 1980s (Tsujimoto et al., 1984) and the caspase family of proteins shown in 1993 to control cell death processes (Vaux, Weissman, & Kim, 1992) have been discovered and extensively studied. Two major routes that have been shown to result in an apoptotic death are the extrinsic pathway and the intrinsic/mitochondrial pathway, both of which consist of an activation phase followed by the execution phase. Caspases (executioner

caspases) are responsible for inducing cellular blebbing (e.g., large vacuoles of cytoplasmic and smaller vacuoles of endoplasmic reticulum (ER) origin) as well as nuclear fragmentation (Liu, Zou, Slaughter, & Wang, 1997; Liu et al., 1998), and can be categorized as initiator or executioner/effector caspases (Riedl & Salvesen, 2007; Timmer & Salvesen, 2007), where caspases 3 and 7 are executioner/effector caspases in both the extrinsic and intrinsic pathways. In the extrinsic pathway, caspases 8, 9, and 10 are initiator caspases. The extrinsic pathway is initiated by an external death signal such as the binding of tumor necrosis factor-alpha (TNF-α), Fas ligand (FasL), or TNF ligand super family member 10 (TRAIL) to receptors on the cell surface. This stimulus triggers the formation of the death-inducing signaling complex consisting of Fas-associated protein with death domain (FADD) (Kischkel et al., 1995), an adaptor protein that bridges the tumor necrosis receptor super family to caspase-8 or 10, which in turn leads to the cytosolic cleavage and activation of effector caspases 3 and 7. In some instances, caspase-8-mediated cleavage of Bid (a Bcl-2 family protein), as well as outer mitochondrial membrane permeabilization (MMP), prior to activation of the executioner caspases are required. The intrinsic or mitochondrial-mediated apoptotic pathway involves the release of cytochrome *c* from the mitochondria caused by the BcL-2 protein family that induces permeabilization of the outer mitochondrial membrane. Cytochrome *c* then binds the apoptotic protease-activating protein 1 (Apaf-1) inducing hydrolysis of dATP. In the absence of an apoptotic signal, Apaf-1 is a monomer containing a caspase recruitment domain on the N-terminal (to which caspase-9 is recruited), cytochrome *c*-binding site at the C-terminus, and a nucleotide-binding and oligomerization site where ATPase is located. Upon activation, oligomerization of APaf-1 occurs creating the heptamer called the apoptosome. Caspase-9 is then recruited to the apoptosome and activated. This initiator caspase activation thus triggers the cleavage of the effector caspases and initiates the signaling cascade leading to apoptosis (Kim, Du, Fang, & Wang, 2005; Liu, Kim, Yang, Jemmerson, & Wang, 1996; Zou, Henzel, Liu, Lutschg, & Wang, 1997). It should be noted that caspase inhibition that is often required for the morphological features of apoptosis, such as nuclear fragmentation and membrane blebbing, can change the morphological appearance of apoptotic cell death to an autophagic or necrotic morphological appearance; thus the initiating stimuli can affect the resulting mode of cell death by inhibition of catabolic enzymes (Galluzzi et al., 2007; Golstein & Kroemer, 2005; Kroemer & Martin, 2005). There is no doubt that apoptosis is a complex and multifaceted form of cell death,

that can occur through multiple mechanisms that result in similar morphological features. Some of these pathways will be presented later as examples with focus on the involvement/role of various cellular organelles. However, more in-depth mechanisms and pathways are beyond the scope of this chapter and presented in other excellent reviews (Strasser et al., 2000).

1.2 Autophagy (Type 2 Cell Death)

Autophagy is recognized as an essential cellular function maintaining homeostasis and aiding cells in adapting to environmentally induced stress conditions such as nutritional deprivation, energy depletion, ER stress, oxidative stress, and hypoxia (Kroemer, Marino, & Levine, 2010; Ryter & Choi, 2013; Ryter, Cloonan, & Choi, 2013; Ryter, Mizumura, & Choi, 2014). It is a slow spatially constricted physiological mechanism that entails a self-digestive, catabolic procedure resulting in turnover and degradation of proteins, damaged cytoplasmic compartments and organelles (Kroemer et al., 2009). Generally it describes the delivery of cytoplasmic components to the lysosomes, which include various subcellular targets modified by ubiquitination or long-lived proteins and major cellular organelles (such as peroxisomes or mitochondria). While short-lived proteins undergo a selective degradation by the ubiquitin-proteasome pathway, most long-lived proteins are thought to undergo degradation through the lysosomes (Mizushima, Ohsumi, & Yoshimori, 2002). Three types of autophagy have been described: macroautophagy, microautophagy, and chaperon-mediated autophagy. Macroautophagy, however, will be the focus of this chapter (and when autophagy is discussed in this chapter, it refers to macroautophagy) as it is the autophagic pathway believed to be responsible for the degradation of the majority of proteins as well as organelles (Mizushima et al., 2002). Autophagy is rapidly induced by starvation by inhibition of the mammalian target of rapamycin pathway. The autophagic mechanism involves the Atg proteins that are required for the formation of the isolation membrane and the autophagosomes (Mehrpour, Esclatine, Beau, & Codogno, 2010; Mizushima, Levine, Cuervo, & Klionsky, 2008; Peltola, Paakkonen, Kallio, Kallio, & Osteomyelitis-Septic Arthritis Study Group, 2010). An example of organelle-specific autophagy is mitophagy, which is regulated by Pink1 and Parkinson protein-2 (Parkin). This process occurs when superfluous or damaged mitochondria are targeted for clearance by polylysine ubiquitylation of mitochondrial outer membrane proteins such as porin, mitofusin, and Miro mediated by Parkin (an E3 ubiquitin ligase), thus resulting in mitochondria displaying Lys63-linked polyubiquitin chains and p62 (an

autophagosomes-binding adapter) aggregation (Ryter et al., 2014;Youle & Narendra, 2011). Fission of mitochondria thus occurs to generate slices manageable for encapsulation and subsequent autophagic processing. As mentioned above, autophagy also maintains cellular protein homeostasis by removing ubiquitinated protein aggregates, a mechanism termed aggrephagy that includes the action of the selective autophagy adaptor NBR1 (neighbor of BRCA1 gene 1) and autophagy cargo adaptor p62 (Ichimura & Komatsu, 2010; Kirkin et al., 2009).

With respect to the relationship between autophagy and cell death, it is important to highlight that autophagic cell death refers to a cell death that occurs with autophagic morphology and does not necessarily mean that the cell death has occurred through autophagy (Galluzzi et al., 2007; Galluzzi, Vicencio, et al., 2008; Levine & Yuan, 2005). In fact, autophagy has been shown to promote cell survival, and cells demonstrating autophagic cell death morphologies have been shown to survive upon withdrawal of the harmful stimuli (Boya et al., 2005; Neufeld & Baehrecke, 2008). Moreover, in a majority of studies where autophagy is inhibited by genetic knockdown of the essential autophagy (Atg) genes, cell death is not blocked, but rather accelerates (Galluzzi, Vicencio, et al., 2008). This indicates that autophagy has a clear pro-survival role, at least as indicated by most studies that have been performed in in vitro models (Kroemer et al., 2009). Indeed, in vivo studies of autophagy are few. However, knockdown/knockout of essential autophagy genes in *Drosophila melanogaster* salivary glands shows reduced cell death, and overexpression of Atg1 kinase leading to induction of autophagy has been shown to lead to death of fat and salivary gland cells in *Drosophila* (Berry & Baehrecke, 2007; Scott, Juhasz, & Neufeld, 2007). In contrast to the engulfment of apoptotic cells that occurs upon clearance by phagocytes, cells dying with an autophagic morphology are not associated with phagocytes (Baehrecke, 2005; Clarke, 1990). Morphologically autophagy does not demonstrate chromatin condensation and is characterized by massive cytoplasmic autophagic vacuolization (Figure 12.2).

The cytoplasmic vacuoles-defined autophagosomes consist of double membranes originating from the ER or cytoplasmic membranes (termed isolation membranes) and contain parts of the cytoplasm destined for bulk degradation in the lysosomes. The elongation of the autophagosomes membrane is dependent on two ubiquitin-like conjugation systems: Atg5-Atg12 and LC3, At8 conjugation system. Atg4B then converts the pro-form of LC3 to its cytosolic form LC3-I. The conversion of LC3-I to its autophagosomes membrane-associated form LC3-II is an initiating

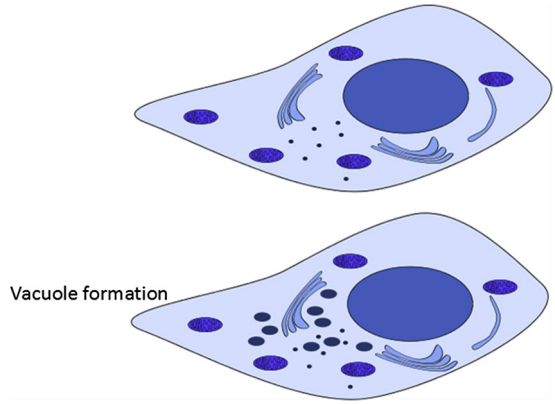

Figure 12.2 ***Schematic representation of autophagy.*** Autophagic morphology is characterized by cytoplasmic vacuole formation. The cytoplasmic vacuoles (autophagosomes) consist of a double membrane originating from the endoplasmic reticulum (ER) or cytoplasmic membranes (termed isolation membranes) and contain parts of the cytoplasm destined for bulk degradation in the lysosomes.

step in autophagy (Ryter et al., 2014). Autophagosomes fuse with the lysosomes in a rate-limiting step creating autolysosomes where the inner membrane and luminal content of the autophagosomes are degraded by acidic lysosomal hydrolases; this final step marks the completion of the autophagic pathway (Gonzalez-Polo et al., 2005; Kroemer et al., 2009; Levine & Kroemer, 2008). Interestingly, when blocking the fusion of autophagosomes with lysosomal, autophagosomes accumulate even when autophagy is inhibited, suggesting that an increase in autophagosomes does not mean that an autophagic pathway is induced and that functional studies are needed for autophagy investigation (Gonzalez-Polo et al., 2005; Klionsky et al., 2012; Levine & Kroemer, 2008). Redistribution of LC3 (into autophagosomes or autolysosomes) is often used as a marker for autophagy; however, this redistribution is not on its own sufficient to prove autophagy. Moreover, NCCD recommends that when this redistribution is observed, it should be described as such (i.e., "vesicular redistribution of LC-3 was observed") rather than an observation of autophagy (i.e., "autophagy was observed") (Kroemer et al., 2009). Furthermore, autophagy has been linked to both apoptosis and necrosis by complex mechanisms. Cells that demonstrate autophagic characteristics can shift to necrotic-like features if early autophagic steps are blocked (Degenhardt et al., 2006; Golstein & Kroemer, 2007; Shimizu et al., 2004). Numerous apoptotic regulators such as p53, caspase-3, and FADD have been

implicated in the cross talk between autophagy and apoptosis. In addition, Beclin-1 (bec-1), a caspase substrate, has been demonstrated to play a key role in the cross talk between apoptosis and autophagy by its interaction with Bcl-2 (Amelio, Melino, & Knight, 2011; Pattingre et al., 2005). The autophagic activity of Bec-1 relies on its disassociation from Bcl-2 that in turn only inhibits Bec-1 when it is localized to the ER. Bcl-XL has also been implicated to reduce the pro-autophagic activity of Bec-1 (Amelio et al., 2011; Maiuri et al., 2007; Pattingre et al., 2005). Indeed, it seems that the role of autophagy is to either contribute to cell death or function as a protective mechanism against acute stress (Kroemer & Jaattela, 2005; Kroemer et al., 2005; Shimizu et al., 2004). With regards to apoptosis, autophagy may occur in a failed attempt to aid cell survival and may have prevented death under milder conditions; while it has been hypothesized that excess autophagy may contribute to apoptosis through unchecked degradative processes (Galluzzi, Vicencio, et al., 2008; Ryter et al., 2014).

1.3 Necrosis (Type 3 Cell Death)

Necrotic cell death is morphologically characterized by cellular swelling (oncosis), swelling of organelles, and early plasma membrane rupture leading to loss of intracellular content (Figure 12.3).

Even though necrosis may be the result of extensive injury or insult to cells, leading to an uncontrolled and accidental mode of cell death, an increasing amount of investigations has recently demonstrated that this mode of death may be regulated by various signaling pathways and catabolic mechanisms (Festjens, Vanden Berghe, & Vandenabeele, 2006; Golstein & Kroemer, 2007). These signaling events have been suggested to involve death domain receptors and Toll-like receptors (TLRs) such as TNFR1, Fas/CD95, TRAIL and TLR3, TLR4 respectively (Kroemer et al., 2009). Investigations involving chemical downregulation (e.g., Nec1, a receptor interacting protein 1 (RIP1) inhibitor) or genetic knockdown of RIP1 have demonstrated that RIP1 is a key player in the signaling pathways mediated by TNFR1, Fas/CD95, TRAIL, and TLR3 (Degterev et al., 2008; Festjens et al., 2006; Holler et al., 2000; Kalai et al., 2002), in particular in the presence of caspase inhibitors. Furthermore, TNF-α-induced ROS production and necrosis can be dampened by rotenone (inhibits respiratory chain complex 1) and inhibited by Bcl-2 (by maintaining mitochondrial integrity) (Golstein & Kroemer, 2007; Schulze-Osthoff, Beyaert, Vandevoorde, Haegeman, & Fiers, 1993). In contrast, death receptor signaling in several cell lines has been shown to lead to activation of caspase-8, which cleaves RIP1 inactivating the pro-necrotic

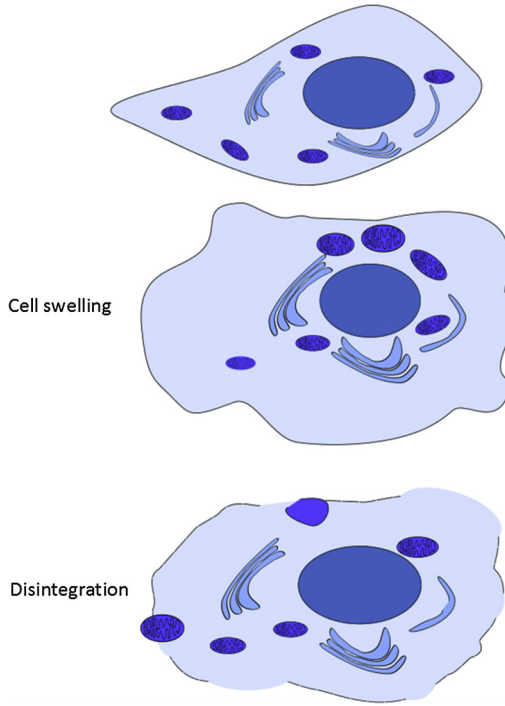

Figure 12.3 ***Schematic representation of necrotic cell death.*** This type of cell death is morphologically characterized by early plasma membrane damage, cellular swelling, perinuclear localization of cytoplasmic organelles and cellular rupture.

activity of RIP1 (Chan et al., 2003; Lin, Devin, Rodriguez, & Liu, 1999; Temkin, Huang, Liu, Osada, & Pope, 2006). The activity of RIP1 has been linked to inflammatory responses, apoptosis, and necrosis as well as stress responses. It has also been shown to be part of the ripoptosome (Feoktistova et al., 2011; Tenev et al., 2011). Moreover, RIP1 has been shown to play a role in cell survival signals by acting as a scaffolding protein upstream of NFκB activation (Lee, Shank, Cusson, & Kelliher, 2004). RIP3 is another member of the RIP family of proteins that has been suggested to be a regulator of programmed necrosis. RIP1 and RIP3 have been shown to form a complex that forms a signaling platform. Indeed, caspase-8 has been shown to result in the association and phosphorylation of RIP and RIP3 that eventually aggregate into complexes resembling microfilaments, referred to as necrosomes. CYLD deubiquitylates RIP in the necrosome resulting in RIP1 activation and recruitment of mixed linkage kinase domain-like (MLKL), which in turn is phosphorylated by RIP3 leading to ROS production and

a programmed necrosis termed necroptosis (Moquin, McQuade, & Chan, 2013; Vanden Berghe, Linkermann, Jouan-Lanhouet, Walczak, & Vandenabeele, 2014). Numerous other signaling mediators and organelle changes have been associated with a programmed form of necrosis (Vanden Berghe et al., 2014) such as uncoupling of mitochondria, MMP, ROS, and/or NO production, bioenergetics crisis, and lysosomal alterations (e.g., membrane permeabilization of ROS production by Fenton reactions). In addition, lipid degradation by the action of phospholipases, lipoxygenases, and sphingomyelinases or nuclear-related changes, such as PARP hyperactivation and NAD^+ hydrolysis as well as activation of cathepsins or calpains due to cytosolic increases in calcium and mitochondrial overload, are all processes that have been shown to regulate programmed necrosis (Festjens, Vanden Berghe, Cornelis, & Vandenabeele, 2007; Kroemer et al., 2009). However, no signaling mediator or biochemical signaling cascade has been exclusively ascribed to programmed necrosis and thus caution in identifying cells as undergoing a programmed form of necrosis should be taken (Kroemer et al., 2009). Often necrosis is classified in the absence of apoptotic or autophagic markers, in particular when cells undergo early plasma membrane damage. Cells with apoptotic or autophagic phenotypes can be shifted to have a necrotic morphology when caspase activation or early steps of autophagy are inhibited (Degenhardt et al., 2006; Golstein & Kroemer, 2007; Shimizu et al., 2004). Nonetheless, in addition to the above-mentioned events demonstrated to occur during programmed necrosis (genetically predetermined), this mode of cell death has also typically been associated with ATP depletion, mitochondrial swelling, perinuclear clustering of cytosolic organelles and loss of calcium homeostasis (Festjens et al., 2006, 2007; Zong & Thompson, 2006). Furthermore, during necrosis, mitochondrial permeability transition (MPT) results in a sudden increase in inner MMP that is caused by opening of a calcium and voltage-sensitive high-conductance channel resulting in a pore formation of both the inner and outer membranes of the mitochondria. As a consequence, a collapse of the ion gradients, the pH gradient, membrane potential, and finally disruption of oxidative phosphorylation (OXPHOS) occurs. Cyclophilin D (CypD) is a mitochondrial matrix protein that can interact with the inner mitochondrial membrane proteins such as ANT and partakes in the MPT. Knockout of CypD induces resistance to necrotic cell death induced by calcium and ROS overload (Golstein & Kroemer, 2007). In addition, inhibition of PARP, CypD and RIP1, calpains, and cathepsins can inhibit necrotic cell death in vivo (Golstein & Kroemer, 2007; Kroemer & Martin, 2005). An early occurrence of these events is essential in determining

the mode of cell death and as apoptotic events require ATP; ATP has been suggested to be a molecular switch between apoptosis and necrosis (Leist, Single, Castoldi, Kuhnle, & Nicotera, 1997; Rasola & Bernardi, 2007). Moreover, the chronological order of events such as changes in ATP, ROS, and calcium can be difficult to assess, as the possibility of feed-forward loops has been suggested to occur and these signals may promote self-destruction (Kim, He, & Lemasters, 2003). The mode of cell death also relies on the model used for investigation; in some models inhibition of caspases is sufficient for inducing necrosis rather than apoptosis, whereas in other models such as immortalized newborn mouse kidney epithelial cells, inhibition of both apoptosis and autophagy is required to induce necrosis. Similarly, in interdigital cells from the limb anlage in mice that lack Apaf-1, a caspase activator, a nonapoptotic, nonautophagic mode of cell death occurs that demonstrates delayed and partial chromatin condensation (Cande, Cecconi, Dessen, & Kroemer, 2002; Chautan, Chazal, Cecconi, Gruss, & Golstein, 1999; Golstein & Kroemer, 2005). In L2929 (connective mouse tissue-derived cells), necrosis induced by TNF-α induces a rapid mitochondrial ROS production that can be inhibited by complex I.

The RIPK1 inhibitor NEC1 has been shown to inhibit necroptosis, but also sometimes apoptosis (due to the involvement of RIP1 in apoptotic signaling), thus inhibitors of RIPK3 or MLKL might be more specific in delineating necrotic pathways. Necrosis can be both regulated and programmed; terminology such as apoptonecrosis, describing a mixed or intermediate cell death mode is discouraged by the NCCD as it may bring more confusion than clarification (Kroemer et al., 2005). However, necroptosis is often used to describe regulated necrotic pathways. Furthermore, molecular events that initiate regulated necrotic pathways such as ferroptosis, oxytosis, netosis, etosis, cyclophilin-mediated regulated necrosis, parthanatos, pyroptosis, and pyronecrosis have also been described (Vanden Berghe et al., 2014). These pathways all result in the same morphological necrotic features but they occur independently or in the presence of inhibitors of RIP1 and RIP3 kinases, in specific cells and under certain conditions such as pathogen infection, cellular stress, or ischemia reperfusion injury (Vanden Berghe et al., 2014). The details of these pathways are discussed in depth elsewhere (Vanden Berghe et al., 2014). As mentioned above, the cellular bioenergetics process (also discussed in section 4 below) plays a crucial role in cellular survival and death. While the role of ATP is well known in such contexts, NAD^+ molecules are energy intermediates that also play an important role in necrosis. MPT leads to excessive loss of NAD^+ resulting

in an active consumption of ATP molecules in order to maintain the electrochemical gradient across the mitochondrial inner membrane. However, this consumption results in a bioenergetics crisis due to disruption in the equilibrium and finally results in programmed necrosis (Kristian et al., 2011; Oka, Hsu, & Sadoshima, 2012). Indeed NAD^+ consumption due to PARP hyperactivation has been purposed to block glycolysis and lead to metabolic breakdown, an effect that was increased by consumption as a result of ATP-dependent NAD^+ synthesis (Virag, Robaszkiewicz, Rodriguez-Vargas, & Oliver, 2013). Although many signaling molecules play various roles in the process of regulated necrosis, this mode of cell death still requires elucidation and it has been suggested that a wider outlook on regulated necrosis than just necroptotic pathways should be applied. Indeed, Vanden Berghe et al. (2014) highlight the progression of what has been and what is today considered programmed necrosis by pointing out that in 2005 necroptosis was assumed to be a regulated mode of necrosis occurring upon TNF-α stimuli and inhibition of caspases, while in 2008 the role of RIP1K was actively discussed, where it was initially proposed to be specific to necroptosis and then shown to be part of apoptosis as well. Then in 2009 the role of RIP3K was elaborated suggesting that as part of the necrosome RIP3K was an essential part in necroptosis, but was then classified as the major initiator as it can trigger cell death independently of RIP1K. Following this, in 2012, MLK was reported to be an essential downstream of RIP3K, both these molecules have now been shown to be part of the inflammasome activation in monocytic and epithelial cells in a cell death-independent manner as well (Vanden Berghe et al., 2014). Programmed or regulated necrosis is closely connected to both apoptosis and autophagy as well as having a high intrinsic interconnectivity; thus it has been suggested that inhibiting exclusive signaling pathways involved in regulated necrosis would not have cytoprotective effects (Galluzzi, Kepp, Krautwald, Kroemer, & Linkermann, 2014).

1.4 Alternative Cell Death

1.4.1 Mitotic Catastrophe

As implied by the name, this mode of cell death occurs during or right after a failed mitosis and can result in both a necrotic or apoptotic phenotype. To this end, the NCCD recommends the terminology, cell death preceded by multinucleation or cell death occurring during metaphase (Kroemer et al., 2009). The morphological changes can include unevenly distributed (between daughter cells) chromosomes or chromosome fragments termed

micronucleation or include multinucleation, meaning the presence of two or multiple nuclei occurring due to an unsuccessful separation during cytokinesis (Candi, Schmidt, & Melino, 2005).

1.4.2 Cornification

This mode of programmed cell death is morphologically and biochemically different from apoptosis and takes place in the epidermis. It results in dead keratinocytes (corneocytes) containing lipids and proteins that are essential for the cornified skin layer. Cornification is considered as a terminal differentiation requiring many of the molecules involved in cell death such as caspases (Candi et al., 2005; Kroemer et al., 2009; Lippens, Denecker, Ovaere,Vandenabeele, & Declercq, 2005).

1.4.3 Anoikis

Ankiosis is an apoptotic cell death that takes place upon loss of attachment to other cells or to the substrate. It involves a classical apoptotic molecular mechanism. The NCCD recognizes this terminology, but stress that it is essential to ensure whether other modes of cell death (such as necrosis or autophagy or a caspase-independent pathway) may occur in vivo upon detachment under special circumstances (Grossmann, 2002; Kroemer et al., 2009).

1.4.4 Excitotoxicity

Depending on the extent of stimuli, excitotoxicity can overlap with both apoptosis and necrosis and it also requires MMP and nitric oxide as critical components to this end it cannot be considered a distinct cell death mode. Excitotoxicity occurs in neurons and when induced leads to calcium overload and initiation of death signals as a result of opening of *N*-methyl-D-aspartate Ca^{2+} permeable channels (Kroemer et al., 2009; Melino et al., 1997; Orrenius, Zhivotovsky, & Nicotera, 2003).

1.4.5 Wallerian Degeneration

Wallerian degeneration is the catabolic process of degeneration of a neuron or axon that occurs without influencing the main cellular body and without the affected neuron actually dying (Kroemer et al., 2009; Luo & O'Leary, 2005; Raff, Whitmore, & Finn, 2002; Sperandio, de Belle, & Bredesen, 2000). In addition to different modes of cell deaths, numerous cell death subroutes have been linked to the action or change of specific organelles, some of which will be discussed in the following sections of this chapter.

2. LYSOSOMAL-RELATED CELL DEATH

2.1 Lysosomal Damage and Related Mechanisms

As the key degradative compartments of the cells, lysosomes and the enzymes residing in these compartments, help to maintain cellular metabolic homeostasis by participating and regulating degradation of heterophagic and autophagic material and thus regulating the turnover of organelles, macromolecules (through autophagy), and endocytosed material (Repnik, Stoka, Turk, & Turk, 2012). In addition, these hydrolases play key roles in cancer progression when released into the extracellular space as an increase in lysosome expression and altered lysosomal trafficking and function has been shown to participate in invasion and angiogenesis (Kroemer & Jaattela, 2005). Thus it is obvious that this multifunctional cellular compartment plays an essential role in cellular life and death scenarios. With regards to cell death, lysosomal membrane permeabilization (LMP) or rupture initiated from stress responses endogenously or exogenously has been shown to lead to the release of lysosomal enzymes with the capacity of inducing caspase-dependent, caspase-independent, apoptosis-like, or necrotic types of cell death (Guicciardi, Leist, & Gores, 2004; Jaattela, 2004), thus coining the term lysosomal cell death (LCD) (Aits & Jaattela, 2013). Indeed, apoptotic hallmarks such as chromatin condensation, plasma membrane blebbing, and phosphatidylserine exposure have been shown to occur in a caspase-independent but cathepsins-mediated manner. Similarly, abolishing heat-shock protein 70-1 (HSP-70-1) in cancer cells or supraoptimal activation of T-cells have been shown to trigger LMP- and cathepsins-mediated cell death in a programmed and apoptosis-like manner independent of mitochondrial outer membrane permeabilization (MOMP) and caspase activity (Kroemer & Jaattela, 2005; Michallet, Saltel, Flacher, Revillard, & Genestier, 2004; Nylandsted et al., 2004).

Lysosomes were initially termed the cells' "recycling bins" and "suicide bags" (de Duve, 1983). Lysosomal rupture was already, in the 1970s, accepted as a powerful cell death inducer (Firestone, Pisano, & Bonney, 1979) and it has been suggested that LMP takes place in most if not all cell death pathways. When investigating the role of lysosomes in cell death, it is essential to establish whether LMP occurs due to cell death or if LMP induces or accelerates the cell death process (Groth-Pedersen & Jaattela, 2013; Johansson et al., 2010). The role of lysosomes in cell death, however, has occasionally been overlooked for two main reasons. First, LMP, does not

necessarily change the ultrastructure of lysosomes (Brunk & Ericsson, 1972) and second, methyl-ketone-based protease inhibitors known to inhibit caspase activity and thus generally linked to caspase-mediated cell death, in fact also inhibit cysteine cathepsins (Guicciardi et al., 2004; Schotte, Declercq, Van Huffel, Vandenabeele, & Beyaert, 1999). LMP can be induced by viral proteins, bacterial toxins, viral cationic peptides (Aits & Jaattela, 2013), as well as lysomotropic agents (Aits & Jaattela, 2013; de Duve, 1983; Firestone et al., 1979). Furthermore, a decrease in lysosomal membrane proteins, LAMP-1 and LAMP-2, or changes in the lysosomal membrane composition have been linked to lysosomal membrane instability and susceptibility to LMP. In support of this notion, it has been suggested that increased cholesterol in lysosomal membranes increases lysosomal membrane stability and attenuates apoptosis (Appelqvist et al., 2011; Fehrenbacher et al., 2008).

Moreover, LMP is intimately linked to ROS production in cases where LMP is induced by oxidative stress that can arise from treatments with heavy metals, certain drugs, etc., or by pathological conditions such as inflammation or neurodegenerative disorders. Indeed, antioxidants and redox regulators protect against oxidative stress-induced LMP. In addition, ROS has been suggested to induce LMP by activating lysosomal Ca^{2+} channels as well as by altering the activity of phospholipase A_2 (Guicciardi et al., 2004). Moreover, hydroxyl radicals have been shown to destabilize lysosomal membranes by lipid peroxidation or by causing damage to lysosomal membrane proteins. This latter manifestation takes place upon diffusion of excess H_2O_2 into lysosomes resulting in Fenton-type reactions leading to production of hydroxyl radicals due to reaction between H_2O_2 and redox-active ion molecules (Guicciardi et al., 2004; Sumoza-Toledo & Penner, 2011).

Even though lysosomal proteases and cathepsins are often demonstrated to be downstream effectors of LCD, in some cases they have been shown to also initiate LMP. Cathepsins have been shown to induce LMP upon minor leakage by cleaving cytosolic proteins involved in maintaining lysosomal stability (Mora et al., 2010; Taha et al., 2005). On the other hand, secondary LMP has been suggested to accelerate and/or amplify cell death. This mode of LMP has been linked to the apoptotic activity of caspases such as caspase-9 in the apoptosome (Aits & Jaattela, 2013; Gyrd-Hansen et al., 2006; Oberle et al., 2010). In addition, both caspase-8 and caspase-7 activation in a TNF-dependent pathway have been shown to result in cathepsins D release. TNF-induced caspase-8 cleavage and subsequent cleavage of Bid to tBid has been shown to activate Bcl-2 proteins Bax and Bak, and lead to MOMP. LMP has been reported to occur in this pathway in a

Bid-dependent manner as the absence of Bid partially inhibits LMP (Aits & Jaattela, 2013; Werneburg, Guicciardi, Yin, & Gores, 2004). It is, however, unclear whether LMP results from a direct effect of Bid or is a result of the bid-induced MOMP. Indeed, the Bcl-2 family members Bim and Bax have both been implicated in LMP. Upon TRAIL treatment, Bim has been shown to recruit active Bax to lysosomes and induce LMP. Moreover, Bax has been shown to induce lysosomal pore formation in isolated lysosomes (Kagedal et al., 2005) in vitro, while Bim has been implicated in acidification that might result in LMP (Ruppert et al., 2012). Cathepsin cleavage of Bid or cathepsin-mediated blockage of Bcl-XL and Mcl-1 can induce MOMP and thus apoptosis-mediated cell death (Aits & Jaattela, 2013).

Another key apoptotic regulator, p53 has been shown to induce LMP (Aits & Jaattela, 2013). Indeed, the recruitment of Ser15-phosphorylated p53 to the lysosomal membrane in an LMP-inducing lysosome-associated apoptosis-inducing protein containing PH and FYVE domains (LAPF)-dependent manner has been demonstrated (Li et al., 2007). Furthermore, it has been shown that LMP can occur in the absence of p53 (Erdal et al., 2005; Nylandsted et al., 2000). Lipids and lipid metabolites have also been implicated in LMP and LCD. Sphingosine-1-phosphate (SIP-1) has been demonstrated to maintain lysosomal function, and loss of SIP results in lysosomal damage as it blocks lysosomal recycling. In addition, excess levels of both sphingomyelin and sphingosine destabilize lysosomes (Mora et al., 2010; Taha et al., 2005). Lipid mediators such as arachidonic acid can be generated through the action of cathepsins D. These mediators as well ROS have been suggested to be responsible for loss of inner mitochondrial membrane damage (Boya et al., 2003; Foghsgaard, Lademann, Wissing, Poulsen, & Jaattela, 2002; Yuan et al., 2002; Zhao, Brunk, & Eaton, 2001).

It has also been demonstrated that micro-tubuli targeting drugs that disrupt the cytoskeleton and cellular trafficking as well as depletion of cytoskeleton-associated motor proteins, induce LMP (Aits & Jaattela, 2013; Broker et al., 2004; Groth-Pedersen, Ostenfeld, Hoyer-Hansen, Nylandsted, & Jaattela, 2007; Groth-Pedersen et al, 2012). As discussed above, numerous triggers can induce LMP and the extent of LMP has been shown to determine the mode of cell death where a burst in LMP is correlated with necrosis and rapid plasma membrane damage (Aits & Jaattela, 2013). In necroptosis and secondary necrosis, LMP is a late event that takes place together with cellular disintegration. In some cases of necrosis, LMP is an early event and leads to excessive release of lysosomal hydrolases (Yamashima, 2012). Minor

lysosomal leakage, on the other hand can initiate the intrinsic apoptotic pathway or a caspase-independent cell death with apoptotic morphology in cells where apoptotic pathways are compromised (Kirkegaard & Jaattela, 2009). Apoptotic caspases can be activated by cathepsins in the cytosol by either being cleaved by cathepsins directly or through cathepsins cleaving the caspase inhibitor E3 ubiquitin-protein ligase XIAP (Aits & Jaattela, 2013). Cathepsin-induced caspases can then induce or accelerate apoptosis in an MOMP-dependent or -independent manner. In addition, cathepsins can cleave several proteins including the caspase substrate PARP (Gobeil, Boucher, Nadeau, & Poirier, 2001) and thus execute cell death (Aits & Jaattela, 2013). However, inhibition of cathepsins only partially inhibits LCD and thus it has been emphasized that the role of other lysosomal enzymes should be further investigated as well as lysosomal second messengers and LMP-mediated lysosomal dysfunction in LCD (Aits & Jaattela, 2013). In addition, it is not known if only a subpopulation of lysosomes is susceptible to undergoing LMP (Kroemer & Jaattela, 2005).

It is evident that lysosomes play a major role in cellular homeostasis a well as demise by partaking in both cell survival and cell death mechanisms including autophagy, apoptosis, necrosis, and necroptosis. Indeed, it was recently shown that dysfunctional lysosomes play a role in neurogeneration as this dysfunction results in impaired constitutive autophagy. Moreover, lysosomal degradation was shown to be required for protective autophagic responses induced by TRAIL, as this step was essential in eliminating caspase-8, since autophagosomes only could continuously sequester caspase-8. Indeed, all forms of autophagy converge on the endocytic pathway as they depend on lysosomal degradation. This further emphasizes the pro-apoptotic role of LMP due to the fact that in addition to releasing lysosomal hydrolases into the cytosol and initiating or accelerating death pathways, this release also impairs the degradative ability of the endocytic compartment and thus weakens the cytoprotective mechanism of autophagy and lowers the apoptotic threshold (Repnik, Cesen, & Turk, 2013).

Moreover, it has been shown that cathepsin inhibitors impair growth, vascularity, and invasiveness in advanced tumors and that tumor invasion and growth are reduced upon genetic suppression of the cathepsins B gene (Joyce et al., 2004; Kroemer & Jaattela, 2005; Mohanam et al., 2001). With regards to alterations in lysosomal trafficking in cancer models, the small GTPases RHOA, RHO-associated, coiled coil containing protein kinase (ROCK), and LIM-domain kinase 1 have been shown to partake in lysosomal redistribution from perinuclear region to the periphery of the cell

and also result in a decreases in lysosomal size (Nishimura, Itoh, Yoshioka, Ikeda, & Himeno, 2002, Nishimura, Itoh, Yoshioka, Tokuda, Himeno, 2003; Nishimura, Yoshioka, Bernard, Himeno, Itoh, 2004). These GTPases are suggested to be acting downstream of RAS, a GTPase often correlated to carcinogenic development and progression (Nishimura et al., 2002, 2003, 2004). The Rab GTPase family has also been shown to control the formation and trafficking of lysosomes along the cytoskeleton and common changes in this family and its downstream effectors in cancer have also been suggested to play a role in altered lysosomal trafficking (Cheng et al., 2004; Lackner et al., 2005). Moreover, inhibition of PI3K has been shown to result in swelling of perinuclear localized lysosomes and the incorrect sorting of cathepsin D to secretory granules. PI3K regulates the stability, size, maturation, and activity of lysosomes, and inhibition of PI3K has been suggested to sensitize cells to LCD pathways (Brown, DeWald, Emr, Plutner, & Balch, 1995).

Considering that LMP often results in cell death, it is rational that cancer cells have development mechanisms to avoid LMP such as exploiting the protective role of HSP70 by expressing this protein in the inner leaflet of the endolysosomal membranes. Indeed, deletion of HSP70 in tumor cells in vitro and in tumor xenografts in vivo has been shown to induce MLP and LCD, and overexpression of HSP70 correlates with a poor prognosis in breast and endometrial cancer patients.

2.2 Relevance for Transfection Carriers

In view of the role of lysosomes in LMP and LCD, it is obvious why current research attempts to exploit these mechanisms for therapeutic purposes. In addition, with evolving progress in the nanotechnology and polymer technology, it may very well be possible to design compounds that more specifically target the endocytic pathway and more efficiently induce membrane destabilization in a specific manner in the digestive system of the endocytic pathway in order to minimize adverse effects (Repnik et al., 2013). In addition, numerous delivery vehicles have been shown to internalize through the endocytic pathway and end up in the endolysosomal compartment. To date, one of the major hurdles of gene delivery systems is the engulfment of delivery vehicles and their cargo in the lysosomes (Durymanov et al., 2012; Forrest & Pack, 2002; Jin, Zeng, Liu, Deng, & He, 2014; Parhamifar, Larsen, Hunter, Andresen, & Moghimi, 2010; Parhamifar, Wu, Andersen, Moghimi, 2014). Furthermore, several studies using polyethylenimine (PEI) report the occurrence of lysosomal damage (Gao et al., 2011; Lin, Jan, Kuo, Hsu, & Lin, 2012) as well as both apoptotic and necrotic cell death depending on

the design of the study and the target disease model investigated (Hall et al., 2013; Larsen et al., 2012; Moghimi et al., 2005; Parhamifar et al., 2010; Symonds et al., 2005). A reoccurring hypothesis regarding the release of polycations from the endolysosomal compartment is the so-called "proton sponge" effect. This hypothesis is based on the high buffering capacity of polycations, such as PEI, that are suggested to result in an increase in lysosomal pH and the proton sponge effect. However, this hypothesis is heavily debated and a recent study making use of lysosomal pH sensors demonstrated that the pH change in PEI-containing lysosomes does not occur and thus the proton sponge effect may not be the main route of escape (Benjaminsen, Mattebjerg, Henriksen, Moghimi, & Andresen, 2013).

In addition, it has been shown that autophagy can play a protective or potentially accelerating role in polycation-induced cell death (Gao et al., 2011; Lin et al., 2012), and it has been demonstrated that the two polycations, PEI and poly-L-Lysine can induce autophagic responses (Gao et al., 2011; Lin et al., 2012). In one study, the autophagic responses upon polycation treatment (up to 3 h) were mainly correlated to the uptake (mainly clathrin mediated endocytosis) and the endolysosomal transport pathway as well as lysosomal damage (Gao et al., 2011). Following polycation removal and 24 h recovery period, mitochondrial damage was accelerated and linked the autophagic responses. In another study, autophagy was suggested to be a protective mechanism initiated upon cellular administration of PEI and poly-L-Lysine (Lin et al., 2012).

Conclusively, cationic polymers and other gene delivery vehicles can be designed to serve purposes that exploit the survival and/or death mechanisms induced through the endocytic pathway or designed to overcome and escape these responses to deliver nucleic acids in a safe and efficient manner. Irrespective of the desired approach, illustrating the subcellular events that take place upon exposure to delivery vehicles in a methodologically accurate manner followed by correct interpretations and nomenclature, is essential for a rational approach. Indeed, the presence of cationic delivery vehicles or other delivery agents and their cargo in the endolysosomal compartment may alter lysosomal trafficking or function and integrity, which could result in initiation or alteration of any of the survival or death pathways where lysosomes play key regulatory effects. As previously mentioned, lysosomal trafficking and cathepsin expression and activity are altered in cancer cells, a fact that may play an important role in investigating the effect of delivery vehicles such as PEI on lysosomes in such models as compared to other target disease models.

In addition, a comprehensive understanding of lysosomal function can help the design, targeting, and delivery of therapeutic agents in the field of cancer nanomedicine.

3. ER-RELATED CELL DEATH

3.1 ER Stress Pathways

ER is the cellular reservoir for calcium and the major site for protein folding (Gaut & Hendershot, 1993). ER-associated degradation will degrade the unfolded and improperly folded proteins, but if high degree of unfolded proteins accumulates in the ER, the unfolded protein response (UPR) will be initiated. Three different pathways are initiated as response to the accumulations of unfolded proteins (Schroder & Kaufman, 2005). Under normal conditions, BiP is bound to the three transmembrane proteins initiating the ER stress pathways (PKR)-like endoplasmic reticulum kinase (PERK), IRE1, or ATF6 (Schroder & Kaufman, 2005). Upon ER stress, BiP dissociates from the transmembrane proteins initiating the UPR. The UPR is initiated by calcium depletion, glucose deprivation, or viral infections among other cellular changes. The UPR is a pro-survival response but prolonged ER stress can result in initiation of an apoptotic response (Jager, Bertrand, Gorman, Vandenabeele, & Samali, 2012). The apoptotic response is initiated when the amount of unfolded proteins in the ER has reached a devastating level. The apoptotic response can further be induced by the release of BiP from ER to the cytosol followed by translocation of BiP to the plasma membrane where it might act as a receptor for pro-apoptotic proteins (Burikhanov et al., 2009).

The IRE1 pathway is initiated when IRE is dissociated from BiP. This leads to dimerization of IRE1 followed by activation of XBP-1 (Jager et al., 2012). XBP-1 is translocated to the nucleus where it promotes transcription of genes involved in the UPR. The IRE1 pathway is usually initiated as a pro-survival signal increasing transcription of proteins involved in the proteasome and has recently been reported to be involved in maintaining the intracellular calcium homeostasis further supporting the association between ER stress and the intrinsic apoptotic pathway (Jager et al., 2012; Son, Byun, Roh, Kim, & Mook-Jung, 2014).

Dissociation of BiP from PERK results in dimerization and auto-phosphorylation of PERK. This is followed by activation of eIF2 which translocates to the nucleus leading to reduced protein synthesis and blocking of protein translation processes resulting in reduced workload of ER (Harding, Zhang, & Ron, 1999).

On activation, ATF 6 translocates to the Golgi apparatus where it is cleaved and a subunit is transported to the nucleus to function as a transcription factor. The ATF 6-initiated pathway is associated with increased transcription of proteins which functions as ER chaperones. This will increase the folding capacity of the ER and help to lower the ER stress (Schroder & Kaufman, 2005).

If the ER stress becomes prolonged, it can be devastating and result in apoptosis. The ER-induced apoptosis pathways are not fully elucidated. In 2000, it was suggested that caspase-12 was responsible for ER stress-induced apoptosis in rats/mice, and activation of caspase-12 has been detected after glucose deprivation (de la Cadena, Hernandez-Fonseca, Camacho-Arroyo, & Massieu, 2014; Nakagawa et al., 2000). However, the gene encoding caspase-12 in humans is not transcribed but caspase-4 was believed to be involved in ER stress-induced apoptosis in humans (Fischer, Koenig, Eckhart, & Tschachler, 2002; Hitomi et al., 2004). In recent years, it has been speculated that the ER stress-induced apoptotic pathway is not ER specific, but merely a pathway where ER stress might lead to changes in the mitochondrial membrane potential and initiation of the intrinsic apoptotic pathway usually associated with mitochondria (Gorman, Healy, Jager, & Samali, 2012). Several ER stress-related proteins have been suggested to be involved in ER stress-induced apoptosis, but also proteins from the Bcl-2 family are reported to play essential roles in the ER stress-induced cell death (Logue, Cleary, Saveljeva, & Samali, 2013). C/EBP homologous protein (CHOP) is one of the important players in ER stress-induced apoptosis. CHOP is a pro-apoptotic protein and helps sensitizing cells to apoptosis by downregulating the transcription of genes involved in apoptosis or in oxidative stress. Among the genes where transcription is reduced are pro-apoptotic members of the Bcl-2 family (Oyadomari & Mori, 2004)

Measuring the initiation of ER stress pathway can be investigated by checking up- or downregulation of expression of proteins involved in the pathways. This can be executed by western blotting of whole cell lysates (Qi, Yang, & Chen, 2011). As an example upregulation of the pro-apoptotic protein CHOP, which indicates an ER stress-induced apoptotic pathway is initiated after addition of silver nanoparticles (NPs) (Zhang et al., 2012). After initiating ER stress-induced apoptosis, the activation of caspases can be measured using a synthetic peptide substrate, which after cleavage will fluoresce. The fluorescence is quantified by flow cytometry or other methods which detect fluorescence (Mack, Furmann, & Hacker, 2000). However, it is important to note that there can be unspecific cleavage of the caspases, which can lead to a false positive readout.

3.2 The Role of Calcium Levels

The ER functions as the main calcium storage of the cell and a depletion of calcium will result in ER stress. Calcium binds to several of the ER chaperones which are required for proper protein folding. The release of calcium from the ER mainly occurs through the inositol 1,4,5-triphosphate receptors (IP3R) and ryanodine receptors (Sammels, Parys, Missiaen, De Smedt, & Bultynck, 2010).

During normal conditions in the cell, small amount of calcium is released to the mitochondria, but at ER stress the amount increases and can result in opening of the permeability transition pore (PTP) and prolonged opening will result in depolarization of the mitochondria initiating the intrinsic apoptotic pathway (Bernardi & Rasola, 2007). An increased intracellular calcium level could not only induce apoptosis, but depolarization of a large fraction of the mitochondria can result in necrosis. This occurs after ATP depletion where the energy level has been reduced to such a degree that the apoptotic pathway cannot be initiated (Rasola & Bernardi, 2011). The calcium flux between ER and mitochondria occurs at the mitochondria-associated membrane (MAMs) and is controlled by the properties of IP3Rs. PERK has been reported to be located at the MAM where calcium ions can be transported from ER to mitochondria and increased activation of PERK could lead to higher level of calcium in mitochondria resulting in opening of the PTP (Logue et al., 2013).

BiP is an important regulator of the switch between ER stress-induced cell death and maintaining the calcium homeostasis in the ER. It has been demonstrated that BiP can prevent apoptosis after treating cells with the apoptosis inducer ionomycin, which is a calcium ionophore. Suppressing BiP will lead to calcium depletion from ER and eventually result in cell death through depolarization of mitochondria (Smaili et al., 2013). The calcium flux between ER and the mitochondria is highly important for the cell fate since a very high calcium level in the mitochondria will result in opening of the PTP and release of cytochrome c and other pro-apoptotic proteins into the cytosol (Schild, Keilhoff, Augustin, Reiser, & Striggow, 2001).

3.3 Transfection Carriers and ER Stress Responses

Cellular calcium levels can be measured with the use of fluorescent probes, which bind to free calcium ions. A possible calcium release from ER can be measured by fluorescence microscopy or flow cytometry using one of the

commercially available fluorophores. However, it is important to consider the advantages and disadvantages of the various indicators. Some of them might introduce a buffering capacity or induce cytotoxicity in the cells (Dewitt, Laffafian, Morris, & Hallett, 2003).

In order to measure if addition of NPs to the cells could result in a calcium response, live-cell imaging can be performed after loading the cells with an indicator that can detect free calcium in the cytosol. Also non-optical measurements of intracellular calcium levels such as electrophysiology and calcium-selective electrodes can be used (Takahashi, Camacho, Lechleiter, & Herman, 1999). An increase in cytosolic calcium after addition of NPs to PC12 cells has been measured with the use of microscopy after addition of a calcium sensor (Huang, Ou, Hsieh, & Chiang, 2000), and a fluorescent probe was also used to detect an increase in cytosolic calcium level after addition of PEI-coated beads to muscle cells (Zhu & Peng, 1988).

4. MITOCHONDRION-RELATED CELL DEATH ON POLYCATION TREATMENT

Normal growth and development of multicellular organisms are dependent on tight regulation of apoptosis. Mitochondria are small double-membrane organelles that are interconnected in various cell signaling pathways controlling cellular life and death (Tait & Green, 2012). A number of vital events of apoptosis occur in mitochondria and/or as a direct result of mitochondrial regulation. In response to various cellular stresses, the pro-apoptotic proteins BAX and BAK are activated and embedded in mitochondria, resulting in MOMP and the release of mitochondrial pro-apoptotic proteins (Moldoveanu et al., 2013; Parsons & Green, 2010). The release of these proteins, such as cytochrome *c*, from the mitochondrial intermembrane space leads to caspase activation and the biochemical execution of cells, characterized by morphological changes and nuclear condensation (Moldoveanu et al., 2013; Parsons & Green, 2010; Tait & Green, 2012). Recent studies have shown that PEI-mediated cytotoxicity is generally characterized by cell death through a mixture of apoptotic and necrotic pathways, interconnected with autophagy responses and mitochondrial dysfunction (Gao et al., 2011; Grandinetti, Ingle, & Reineke, 2011; Hall et al., 2013; Hunter & Moghimi, 2010; Larsen et al., 2012; Lin et al., 2012; Moghimi et al., 2005; Symonds et al., 2005). Polycationic vectors, such as PEI, have previously been shown to induce the release of cytochrome *c* from mitochondria (Hunter & Moghimi, 2010; Moghimi et al., 2005). This

is thought to occur as a result of the formation of nanoscale pores by the polycations in the outer mitochondrial membrane, allowing cytochrome *c* to leak into the cytosol, activating pro-apoptotic caspases and programmed cell death.

Cellular activity depends on the continuous availability of free energy for the execution of numerous energetically unfavorable biochemical reactions. In the majority of healthy cells, mitochondria produce the bulk of the intracellular energy molecule ATP through the process of OXPHOS, providing energy to drive biosynthetic reactions (Babcock & Wikstrom, 1992; Hatefi, 1985; Mitchell, 2011). ATP production by OXPHOS is achieved through tight coupling between the activities of the electron transport system (ETS) and the F_0/F_1-ATP synthase. Four protein complexes (CI–CIV) constitute the ETS, transferring electrons through a series of redox reactions, resulting in the reduction of oxygen to water by CIV (also known as cytochrome *c* oxidase) (Hatefi, 1985). Importantly, electron flow through the ETS complexes is interconnected to the capability of CI, CIII, and CIV to translocate protons across the inner membrane and into the intermembrane space (Babcock & Wikstrom, 1992; Hatefi, 1985). This action generates the proton gradient in the intermembrane space and the mitochondrial membrane potential ($\Delta\Psi$) across the inner membrane (Mitchell, 2011). The F_0/F_1-ATP synthase can utilize the free energy in the proton gradient to drive the production of energy-rich ATP molecules from adenosine diphosphate (ADP) and inorganic phosphate (Itoh et al., 2004). Inadequate mitochondrial ATP production can result in bioenergetic crisis and subsequent cell death. It has been demonstrated that the extent of ATP depletion directs the type of cell death taking place, as mild decline in ATP levels can result in apoptosis, whereas excessive ATP depletion initiates necrosis (Golstein & Kroemer, 2007; Hartley, Stone, Heron, Cooper, & Schapira, 1994; Izyumov et al., 2004; Lieberthal, Menza, & Levine, 1998). It was recently shown that PEI impairs mitochondrial OXPHOS in a biphasic manner (Hall et al., 2013). First, PEI uncouples mitochondrial respiration by reducing the integrity of the mitochondrial inner membrane, resulting in increasing proton leak across the membrane. Second, PEI impairs the activity of the ETS through a potent inhibitory effect on CIV (cytochrome *c* oxidase) (Hall et al., 2013). These events result in dissipation of the $\Delta\Psi$ and cessation of the proton gradient, resulting in diminished OXPHOS. Subsequent to these changes, PEI was found to reduce intracellular ATP levels in a concentration-dependent manner, a phenomenon which was partly due to plasma membrane damage and ATP leak-out of cells (Hall et al., 2013). Accordingly, it is highly

conceivable that the appearance of either necrotic and/or apoptotic cell death processes following PEI exposure could be directed by the magnitude of bioenergetic crisis and the extent of the intracellular ATP depletion. Hence, it is becoming increasingly clear that PEI vectors are severe bioenergetic poisons, raising serious concerns with regard to the safety issues of cationic gene carriers during transfection procedures. Comprehensive studies into mitochondrial function and cellular bioenergetics following polycation exposure are therefore among the most important aspects of toxicological concern during the design of improved carriers of genetic material.

High-resolution respirometry (OROBOROS oxygraph-2k, Innsbruck, Austria) is a highly advanced and useful instrument for detailed investigation of the effects of toxic compounds on the activity of mitochondria as structurally intact organelles in intact cells (Gnaiger, 2001; Hall et al., 2013; Pesta & Gnaiger, 2012). In this section, we provide examples of the detrimental effects of 25-kDa branched PEI (25k-PEI-B) on mitochondrial function in the human nonsmall cell lung carcinoma cell line H1299, a convenient cellular model previously used in the study of PEI-mediated mitochondrial impairment (Hall et al., 2013). Real-time investigations into the time-dependent effect of cellular exposure to 25k-PEI-B (25 μg/mL) on mitochondrial function were performed in growth medium at cellular density of 2.5×10^5 cells/mL at 37 °C. The phosphorylation control protocol was used, allowing for dynamic analysis of different respiratory states (ROUTINE, LEAK, and ETS capacity) in intact cells through the additions of plasma membrane-permeable compounds (Pesta & Gnaiger, 2012). The first respiratory state to be investigated was ROUTINE respiration, monitored at a steady state of cellular oxygen consumption in growth medium. This defines the physiological coupling state of mitochondrial respiration and is closely linked to cellular energy demands (Pesta & Gnaiger, 2012). Subsequent to stable ROUTINE respiration, 25 μg/mL of 25k-PEI-B or H_2O (control) were added to the cells for 2, 5, 30, or 60 min. The 25k-PEI-B was found to affect ROUTINE respiration in a biphasic manner, consistent with previous observations at lower concentrations (Hall et al., 2013). Following the first 2–5 min of PEI exposure, ROUTINE respiration increased sharply; however, with longer incubation times (30 and 60 min) ROUTINE respiration was gradually and effectively diminished (Figure 12.4(A)). Following investigation of ROUTINE respiration, oligomycin (2 μg/mL) was added to the cells to inhibit the mitochondrial F_1/F_0 ATP-synthase. This gives a measurement of mitochondrial LEAK respiration which is independent of ADP phosphorylation and is mainly due

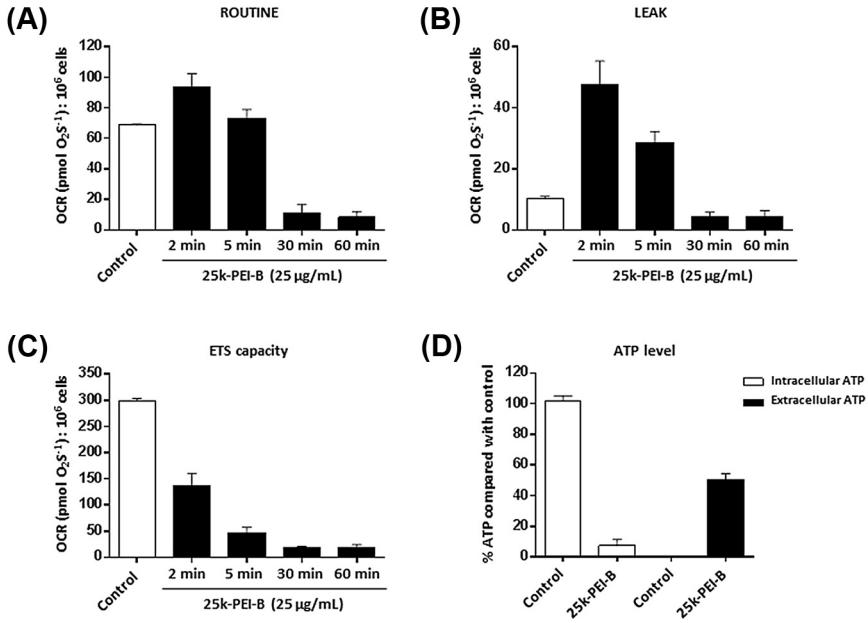

Figure 12.4 ***The time-dependent effect of 25k-PEI-B (25 µg/mL) on respiratory states.*** (A) ROUTINE respiration, (B) LEAK respiration, (C) ETS capacity (indicated as the oxygen consumption rate; OCR) in H1299 cells. Panel (D) shows a comparison of intra- and extracellular ATP levels in H1299 cells following 1-h exposure with 25k-PEI-B (25 µg/mL). PEI, polyethylenimine; ETS, electron transport system.

to proton leak over the mitochondrial inner membrane (Pesta & Gnaiger, 2012). Notably, rise in ROUTINE respiration can be a reflection of the cells' attempt to compensate for increased proton leak. Indeed, LEAK respiration was found to increase in parallel to ROUTINE respiration at early time points (2–5 min) and also to gradually decline at later time points (30 and 60 min) (Figure 12.4(B)). Thereafter, the maximum activity of the ETS was investigated through titrations (0.5 µM) with the protonophore, carbonyl cyanide *m*-chlorophenylhydrazone to obtain maximal respiratory flux (Pesta & Gnaiger, 2012). Addition of 25k-PEI-B had rapid time-dependent inhibitory effect on the ETS system (Figure 12.4(C)), consistent with the fact that 25k-PEI-B acts as a potent inhibitor of cytochrome *c* oxidase (CIV) within the ETS (Hall et al., 2013). Finally, a measurement of residual oxygen consumption (ROX) was obtained through addition of CI inhibitor (Rotenone, at 0.5 µM) and CIII inhibitor (Antimycin-A, at 2.5 µM) (Pesta & Gnaiger, 2012). The respiratory states were corrected for oxygen flux due to ROX and instrumental background. Calibration of the instrument was

performed daily with air-saturated medium. DatLab software (OROBOROS instruments) was used for data acquisition and analysis (Gnaiger, 2001; Pesta & Gnaiger, 2012). Moreover, cellular exposure to 25k-PEI-B (25 μg/mL) for 1 h resulted in excessive intracellular ATP depletion and extensive translocation of ATP over the plasma membrane (Figure 12.4(D)), further demonstrating its toxic effects on intracellular energy homeostasis.

Collectively, these experimental examples clearly demonstrate the detrimental effects that cationic vectors exert on mitochondrial activity and the severity of the following bioenergetic crisis. Future design of improved cationic NPs for transfection purposes should aim at minimizing the observed detrimental effects caused by the polycations on bioenergetic processes and intracellular energy homeostasis.

5. CELL DEATH-ASSAY DESIGN, CONSIDERATIONS, AND INTERPRETATIONS

In addition to appropriate and consistent terminology regarding cell death, accurate choice of methods, correct experimental designs, and appropriate interpretations of readouts in a standardized manner are essential for determining cell death processes. Furthermore, when investigating the cytotoxicity of delivery vehicles, considerations regarding the characteristics of the delivery vehicles investigated must be taken into account when designing, performing, and interpreting cytotoxic studies. With regards to the first consideration (of executing and correctly interpreting cell death investigations), the NCCD has published guidelines regarding the most common assays used to determine cell death, their advantages, drawbacks, and common misconceptions in the literature (Galluzzi et al., 2009). The second consideration, the characteristics of the delivery vehicle, is a field that requires interdisciplinary collaboration illuminating the additional pitfalls and interferences potentially imposed by the delivery agents.

Cell death mechanisms are complex and heterogeneous processes that can involve separate overlapping signaling pathways leading to different morphologies. Mixed morphologies have also been reported which might be the result of several pathways being initiated simultaneously. Due to the fact that to date no specific molecular signaling event has been identified that demonstrates a point of no return, i.e., cell death, the NCCD has recommended the three morphological characteristics explained above to be used as parameters for when a cell is considered dead (Galluzzi et al., 2009). One of these characteristics is loss of membrane integrity. However,

polycations such as PEI have been suggested to induce plasma membrane pore formations that could indeed result in cellular influx of cell impermeable dyes. The questions that arise are then; what happens if this is merely an initial effect of the interaction of the delivery vehicle with the cell? Also, what happens upon removal of the polycations? One could speculate that depending on the extent of pore formation and membrane damage, two possible scenarios could take place. If the insult to the membrane is extensive, the cell would most likely die. On the other hand, if the insult is short-lived or the damage is not extensive, the cell might try to recover from it by initiating repair and survival responses. Thus, careful consideration and several assays are required to investigate this phenomenon. LDH assay and PI influx are commonly used assays for end point responses, however PI is a small molecule and only minor pore formation is required to detect its influx by sensitive methods such as FACS. Continuous treatment of cells with the polycations would naturally lead to increased pore formation and detrimental plasma membrane damage, however, the point of no return might be more difficult to determine purely by this method. Accordingly, the NCCD recommends that in determining cell death mechanism and modalities several assays examining the same event in different supportive ways should be applied. In contrast to PI (or similar dyes), LDH is a rather large molecule and though the cell may recover after minor LDH release, a more extensive LDH response would most likely demonstrate cell death or point of no return. A combination of these two assays alongside morphological assessment of the cells may be a good starting point for determining the time- and concentration-dependent cellular tolerance in response to polycations and help shed light on the kinetics of reaching the point of no return. However, although LDH assay can provide a rough temporal assessment of cell death, it cannot discriminate between the different modes of cell death and this, in this manner, is not very descriptive. In addition, it is well known that LDH can degrade over time or be influenced by altered pH or certain cell culture medium components (Galluzzi et al., 2009; Parhamifar, Andersen, & Moghimi, 2013). With regards to polycations (or other types of delivery vehicles and/or drugs), they can interfere with the readout; thus proper experimental controls are needed to exclude this possibility (Parhamifar et al., 2013). Another enzymatic assay often employed in the field of gene delivery is the MTT assay or its improved versions, MTS and WST-1. Basically these assays monitor substrate conversion by the action of mitochondrial enzymes. However, many drawbacks of these assays have been revealed such as distorting effects caused by

growth medium, fatty acid, and serum albumin (Huang, Chen, & Walker, 2004). In the MTT assay, cells have to be lysed in order to measure the enzymatic activity and the formed cytosolic formazan crystals, that are cytotoxic even in minimal amounts, have to be solubilized overnight. In contrast, the MTS and WST assays are optimized to be less toxic and do not require cellular lysis. However, the conversion of MTT/MTS/WST-1 that are all dependent on the activity of mitochondrial dehydrogenases may result from changes in the cell metabolism that do not have to correlate with the number of viable cells (Galluzzi et al., 2009). In addition, high cell density and medium overconsumption can result in underestimation of viable cells as these factors contribute to shutdown of mitochondrial function (Kroemer et al., 2009).

Exclusion dyes such as PI or 7AAD are often used together with Annexin V staining that recognizes phosphatidylserine in FACS or microscopy analysis. Simultaneous measurement of PI or 7AAD and Annexin V provides a quantitative readout that can be monitored in a time-dependent manner in live or fixed cells. Annexin V staining has long been accepted as a marker for early apoptosis and the progression of single-stained Annexin V cells to becoming double-stained Annexin V and PI has classically been correlated to a progression from early apoptosis (prior to DNA fragmentation and plasma membrane damage) to late apoptosis/secondary necrosis. In contrast, cells that over time directly progressed to double-stained conditions or PI positive and Annexin V negative staining are typically considered necrotic. However, PS exposure has been shown to take place independent of apoptosis, and excessive plasma membrane damage also allows Annexin V access to the inner part of the plasma membrane. Moreover, PS exposure may be compromised in autophagy-deficient cells (Galluzzi et al., 2009). On a technical note, it is also essential to collect the growth medium from the treated cells (as well as PBS from the washing steps prior to trypsinization) in order to capture potentially detached cells. These cells require proper centrifugation speeds, higher than typically used for harvested cells as they are lighter. Moreover, severely damaged cells may not be able to retain staining, thus a proper concentration- and time-dependent analysis must be performed for accurate assessment. Again, with regards to polycations delivery vehicles that can cause membrane pore formations, interpretation of the double-stained cells must be performed with caution. The mode of cell death can also be investigated by visualization techniques such as electron microscopy. In general, microscopy methods (light and fluorescent microscopy) offer rather inexpensive ways of visualizing cell death and morphological changes. However, these methods suffer from

being prone to subjectivity of the investigator and in some cases also might underestimate the extent of cell death and cell death process or focus on rare events. However, many of the drawbacks can be overcome to a large extent with time-lapse live imaging and the methods can overall provide a comprehensive tool for single-cell spatiotemporal investigations of specific cellular events. A combination of microscopy and biochemical quantitative methods is thus essential. In addition to these screening methods, more in-depth studies should be performed to unravel the biochemical features occurring prior to cell death. Such methods involve mitochondrial membrane potential, caspase activity, ROS production, and cleavage or modification of apoptotic and antiapoptotic proteins, cytochrome *c* release, nuclear condensation or fragmentation, cytosolic leakage of lysosomal proteins, etc. Unraveling the specific cascades resulting in various types of cell death accounts for an essential step with regards to therapeutic progression. This includes taking into consideration the target model, something that also is highly relevant for gene therapy purposes too. Cytofluorometry has been suggested the most convenient assay for measuring cell death on a collective single-cell basis (Galluzzi et al., 2009). Several changes related to cell death may be measured quantitatively and simultaneously including ROS production, caspase and cathepsin activity, mitochondrial MPT as well as morphological changes and dye exclusion assays. Flow cytometric assays can measure 10–12 different parameters in both fixed and live cells and are further high-throughput adjusted by 96-well formats. However, protocol compatibility and accuracy with regards to proper settings avoiding false positive or false negative readouts due to inaccurate dye separation are essential to consider.

Investigating the potential cytotoxic profile of a delivery vehicle and/or the potential desired cytotoxic response from the cargo, includes taking into consideration which molecular events are most prominent in the target model, so they can be overcome or induced, respectively. This can be applied, for example, in tumor cells that often demonstrate resistance to apoptotic induction but are susceptible to necrotic triggers (Galluzzi et al., 2009).

Irrespective of the method used for illustrating the mode of cell death and the signaling preceding it, general considerations including technical and biological variations, such as the sensitivity of the assay used, the range of detection, assay throughput, whether the assay is quantitative, qualitative, or semiquantitative should all be part of the investigative design. Numerous methods, their drawbacks and strengths are collected and discussed by the NCCD and provide an excellent guideline for cell death studies (Galluzzi et al., 2009). Similarly, detailed guidelines on investigating and

accurately interpreting autophagy are provided elsewhere (Klionsky et al., 2012; Parzych & Klionsky, 2014; Tabata, Hayashi-Nishino, Noda, Yamamoto, & Yoshimori, 2013).

6. SAFER DESIGN OF POLYCATIONIC SYSTEMS

Recent advances in gene therapy have resulted in the development of a series of new promising polycationic polymers that make use of original polycationic structures such as PEI. These structures have been modified in various ways aiming to diminish cytotoxicity, while preserving or increasing gene delivery efficacy (Lachelt et al., 2014; Salcher et al., 2012; Troiber et al., 2013; Zintchenko, Philipp, Dehshahri, & Wagner, 2008). However, the molecular basis of their safety improvement remains to be elucidated. In addition to these developments, other strategies have been employed making use of layer-by-layer (LbL) (Ariga, Hill, & Ji, 2007; Ariga, Lvov, Kawakami, Ji, & Hill, 2011; Ariga, McShane, Lvov, Ji, & Hill, 2011; Decher, 2012) and simple polymer coating methodologies (Wu et al., 2014).

6.1 LbL Approach

The LbL theory is based on the concept that the formation of ultrathin multilayer films is driven by the ionic attraction between opposite charges (Ariga et al., 2007; Ariga, Lvov, et al., 2011; Ariga, McShane, et al., 2011; Decher, 2012). This easy, inexpensive, and versatile nanofabrication technique has gained an increased importance in the past 20 years. What is more important, surface engineering by polymers can modulate pharmacokinetics and biological performance of NPs (Moghimi, Parhamifar, et al., 2012; Petros & DeSimone, 2010). The LbL approach can be used for the noncovalent modification of the positively charged PEI, which is able to form electrostatic interaction with nucleic acids and other negatively charged macromolecules. For instance, Elbakry et al. (2009, 2012) has used the LbL approach to attach PEI to the surface of gold nanopartices (AuNPs), then to coat these PEI-AuNPs with siRNA (Elbakry et al., 2009) or DNA (Elbakry et al., 2012), followed by another layer of PEI. Both studies showed decreased cytotoxicity compared with the "naked" PEI. However, numerous biochemical and biophysical aspects have to be considered and taken into account during the experimental design and data interpretation. First, the choice of core material is very important, and can have an effect on the final toxicological profile. For example, it was shown that noncovalent binding of 25-kDa branched PEI to the surface of mesoporous silica NPs induced considerable toxicity

depending on cell type and, which was based on the MTS assay (Xia et al., 2009), while PEI-coated polystyrene (PS) NPs did not show any cytotoxicity (Hudzech et al., unpublished data). Second, according to the traditional LbL approach, the electrolytes of the different layers are applied in excess, which is washed away after each equilibration step (Decher, Hong, & Schmitt, 1992). These systems often operate at the top plateau of the adsorption isotherm (i.e., particles with maximal surface coverage on the core molecule) (Figure 12.5). However, the investigation of adsorption isotherm is beneficial, and may reveal some interesting properties. For instance, the adsorption isotherm might differ from the traditional Langmuir profile. For instance, the adsorption isotherm of poloxamine 908 on the surface of polystyrene lattices showed two plateaus due to a conformational change of the poloxamine 908 on the core surface (Al-Hanbali, Rutt, Sarker, Hunter, & Moghimi, 2006; Hamad et al., 2010). Indeed, we recently investigated the adsorption isotherm of PEI on polystyrene lattices and its effect on cytotoxicity and DNA complex formation (unpublished observations). This study revealed that a PEI-PS NP from the midpoint of the adsorption isotherm could result higher transfection efficiency and lower cytotoxicity than PEI-PS NP from the top plateau of the adsorption isotherm, where the surface coverage of PEI is maximum.

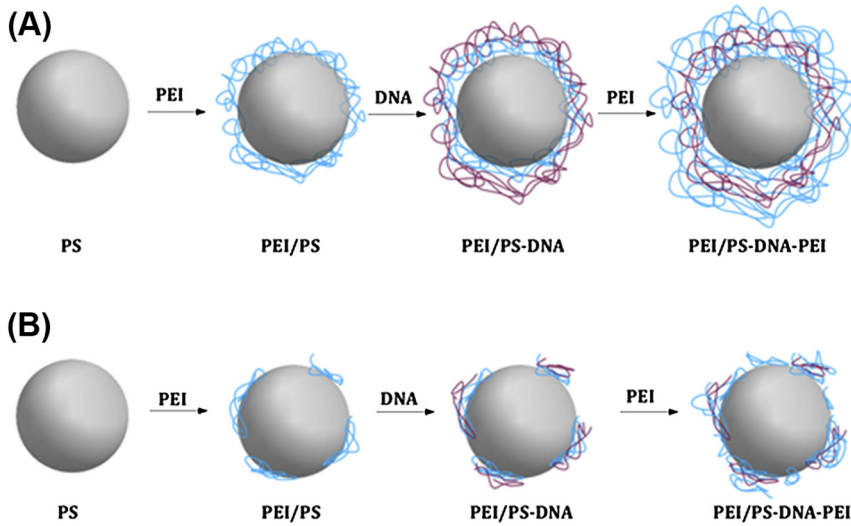

Figure 12.5 ***Layer-by-layer coating of polystyrene (PS) nanoparticles with PEI and DNA.*** Panel (A) represents a case when the surface of a nanoparticle is initially coated with a uniform layer PEI followed by subsequent layers of DNA and PEI, whereas panel (B) shows partial surface coverage with PEI before sequential addition of DNA and PEI. PEI, polyethylenimine.

Finally, there is a need to gain better understanding on the actual mechanism of LbL coating. To the best of our knowledge, there is no published study that investigates the mechanism of complex formation of PEI with core particle and then with DNA or siRNA, and finally with the outer layer of PEI or other positively charged substances. However, this mechanism might not be as simple as it is thought. Our studies indicated that the addition of the second layer of electrolyte, i.e., DNA or siRNA, could lead to the desorption of some of the PEI from the core surface. Accordingly, released PEIs could form complexes with the nucleic acid, which eventually could adsorb back to the core surface as a complex (polyplex), thus forming patchy domains (Hudzech et al., unpublished) or even induce NP aggregation. Although multilayer films have been fabricated using mainly electrostatic attraction as driving force, other interactions, such as donor/acceptor, hydrogen bond, stereocomplex formation, or specific site recognition can further play dominant roles (Decher, 2012). Reproducibility of multilayer formation based on LbL theory depends on the adsorption time, rinsing volume, and surface coverage of functional groups (Decher, 2012), and difficult to achieve. In addition, pH and ionic strength of the solvent can also play important roles. To avoid aggregation due to cross-linking of the particles by polyelectrolyte chains and separation of unbound polyelectrolyte from the coated particle can also be challenging task (Elbakry et al., 2009), which can be reduced by rinsing and washing away the excess of electrolytes after the addition of each layer (Decher, 2012).

6.2 Modulation of Biodegradable Polymeric NPs with PEI

Biopolymer-based NPs are widely used in versatile drug delivery systems because of their novel capabilities, such as easily tailored, encapsulating both hydrophobic and hydrophilic drugs, and biodegradable (Moghimi, Hunter, & Andresen, 2012; Novio, Simmchen, Vázquez-Mera, Amorín-Ferré, & Ruiz-Molina, 2013). There are increasing efforts in the development of simple, highly stable, safe, and biologically compatible polymeric NPs for cellular delivery and controlled release of therapeutic agents (Couvreur, 2013; Feng et al., 2013; Moghimi, Parhamifar, et al., 2012). Surface modification of NPs with different chemical composition and architecture polymers and ligands (peptide, antibody, aptamer) can further increase versatility for high-performance drug delivery (Elsabahy & Wooley, 2012; Naahidi et al., 2013). Among many available biopolymers, polyhydroxyalkanoates (PHAs) are a family of biopolyesters, which are naturally biosynthesized by microorganisms (Chen, 2009; Tripathi, Wu, Chen, & Chen, 2012; Wu, Chen, Li, Xu, & Chen, 2008). Due to their biodegradability, improved

biocompatibility, thermoplasticity, elasticity, piezoelectricity, and optical activity, PHAs have received considerable interest for bioengineering purposes (Chen & Wu, 2005; Wu, Wang, Wang, & Xu, 2010). One notable example is poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx); an amorphous mixture of short- and long-chain random PHA copolymers. Accordingly, PHBHHx has been used widely in medical application, such as cell growth support/scaffolds, implants, and extracellular controlled release matrices, where its degradation products have not induced negative effects on cells (Cheng, Chen, & Chen, 2008; Wu et al., 2013; Xiong, Yao, Zhan, & Chen, 2010). Recently, we have modified the surface of PHBHHx NPs with 10-kDa branched PEI by electrostatic interaction (Wu et al., 2014). PEI coating dramatically enhanced NP–cell interaction irrespective of the cell type, where internalized NPs trafficked along the microtubules as well as ER, and the Golgi complex. Furthermore, PEI-coated PHBHHx NPs were not cytotoxic, since there were no detrimental effects on cell morphology and mitochondrial functionality based on the cell functionality and viability tests (Wu et al., 2014). Accordingly, these engineered NPs may be used as versatile tools for nucleic acid (and other therapeutic agents) delivery to various cell types.

7. CONCLUSIONS

It is evident that despite the progress being made in the field of nucleic acid delivery with polycations, a better understanding of polycation-mediated cytotoxic responses is still required. Cell death processes are dynamic and integrated, and must be investigated in a coordinated manner by considering biochemical responses in a cell-specific manner. Indeed, unraveling the mechanisms of polycation-mediated cytotoxic responses is central for design of safer polymers through assimilated combinatorial and medium/high-throughput chemical/metabolomic approaches, which could substantially improve the delivery of nucleic acids in clinical gene therapy and RNA interference interventions.

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Note: Page numbers with "*f*" and "*t*" denote figures and tables, respectively.

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COLOR PLATES

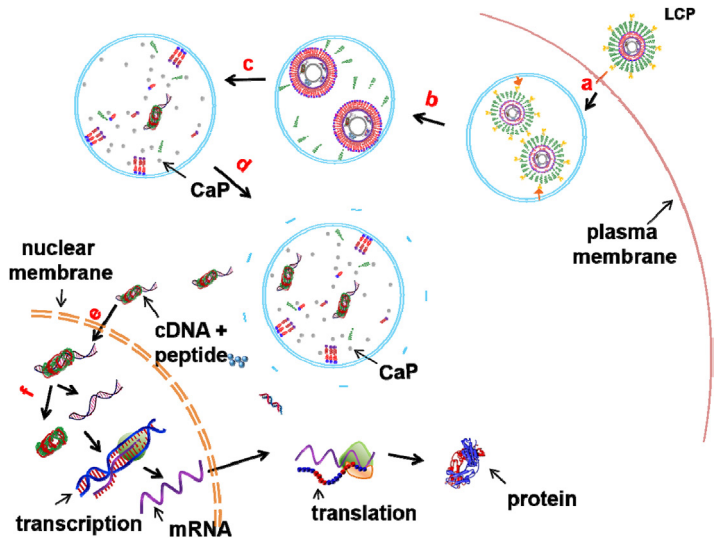


Figure 1.1 ***Proposed mechanism for intracellular delivery of DNA by lipid calcium phosphate (LCP).*** Stepwise scheme for nonviral acid-sensitive vector (LCP), in which the vector is internalized through receptor-mediated endocytosis, destabilized as endosome's pH decreases and releases the DNA–peptide complex into the cytoplasm. The DNA–peptide complex enters the nucleus through the nuclear pore, where it dissociates and releases free DNA, which is transcribed to mRNA, migrates to the cytoplasm to be translated, and results in desired protein synthesis (Hu et al., 2013). *Original figure was prepared by Bethany DiPrete.*

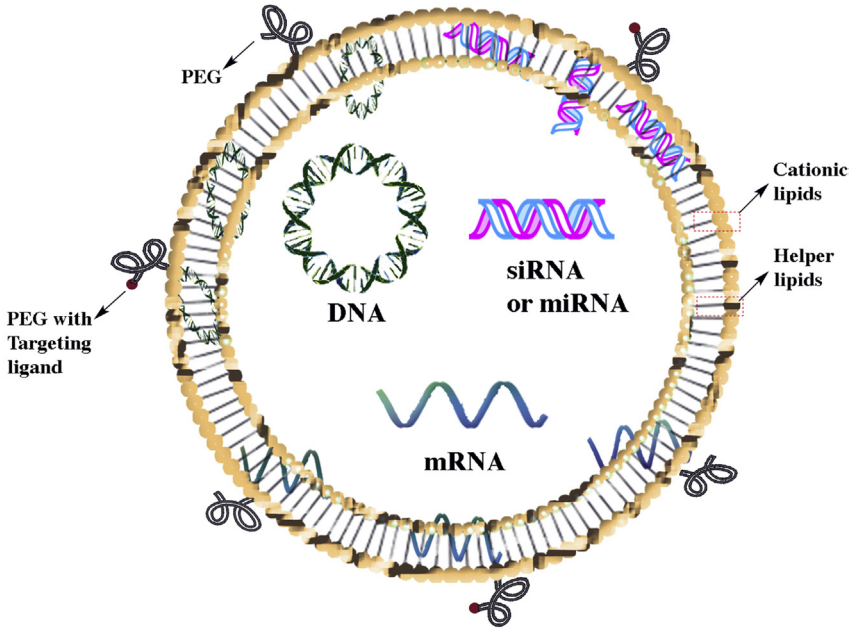


Figure 2.1 Scheme of a lipid nanoparticle (LNP) formed by lipids (yellow), helper lipids (brown), and polyethylene glycol (PEG). Lipids condense and stabilize nucleic acids, which promote the stabilization of LNP.

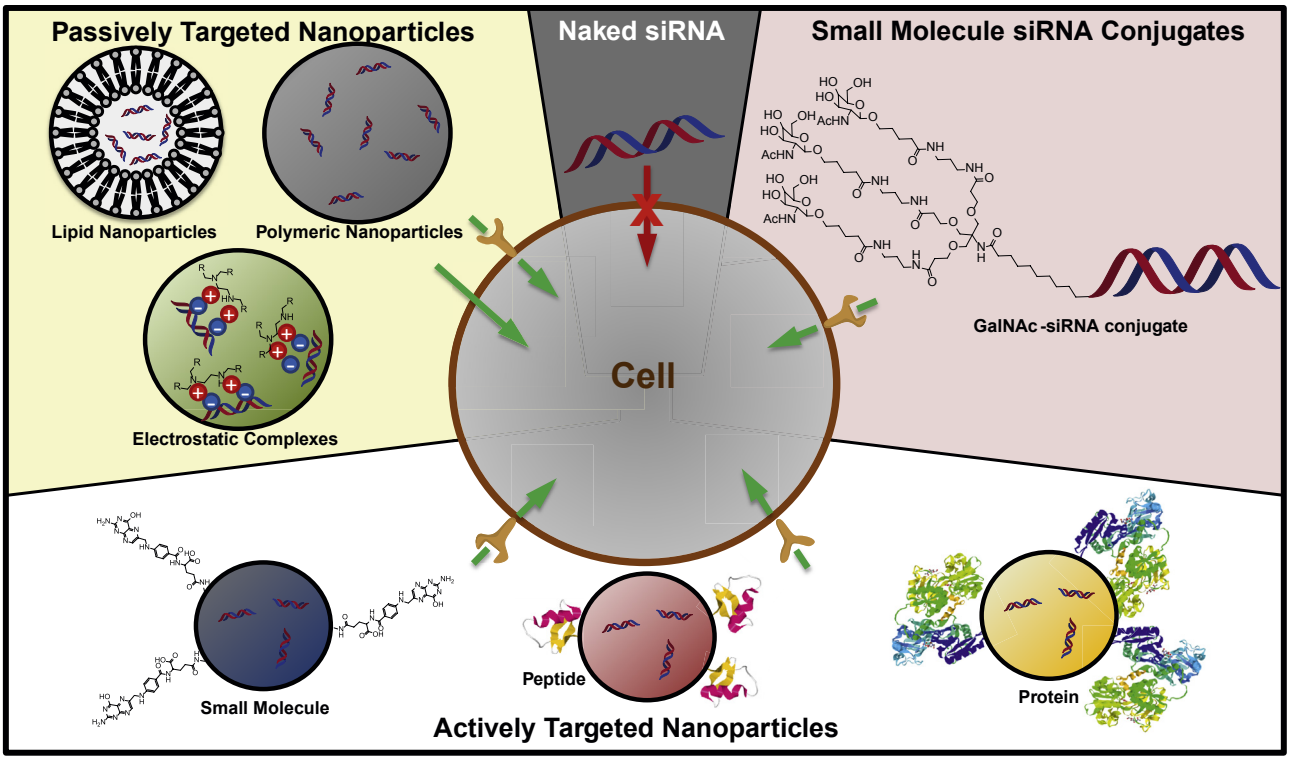


Figure 3.1 ***Strategies to improve siRNA delivery.*** siRNAs, which are large, hydrophilic, and anionic, cannot easily cross the cell membrane by themselves. siRNA delivery can be improved with nanoparticles or conjugates that actively target ligands on the outside of the cell, or by nanoparticles that use natural interactions with the body (e.g., serum proteins) to passively target the cell of interest.

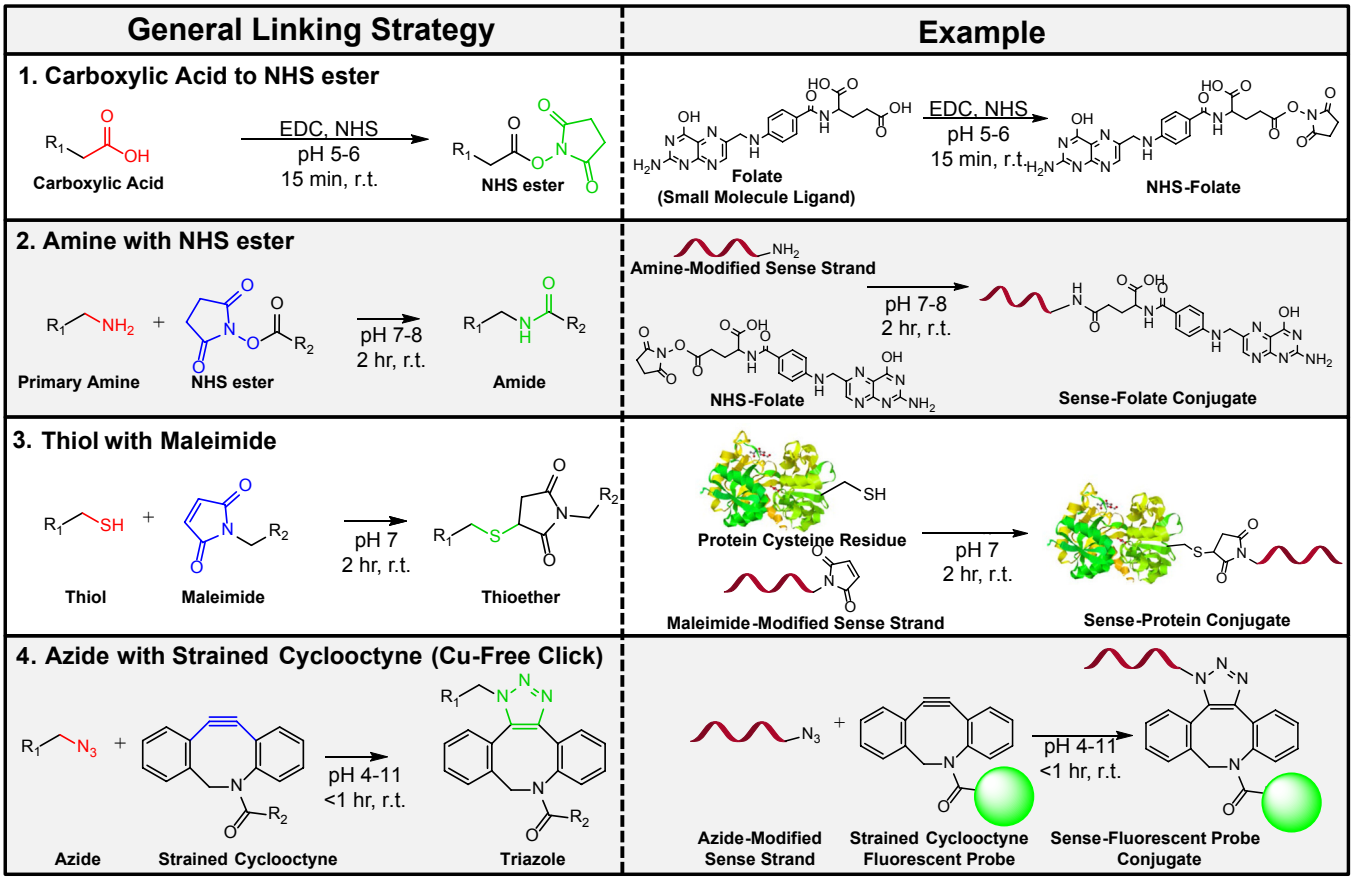


Figure 3.2 ***Chemical reactions to synthesize active siRNA conjugates.*** Conjugation reactions must not denature the targeting ligand, affect siRNA stability, or prevent siRNA loading into RISC. Notably, many synthetic schemes in use today require modification for every new targeting ligand.

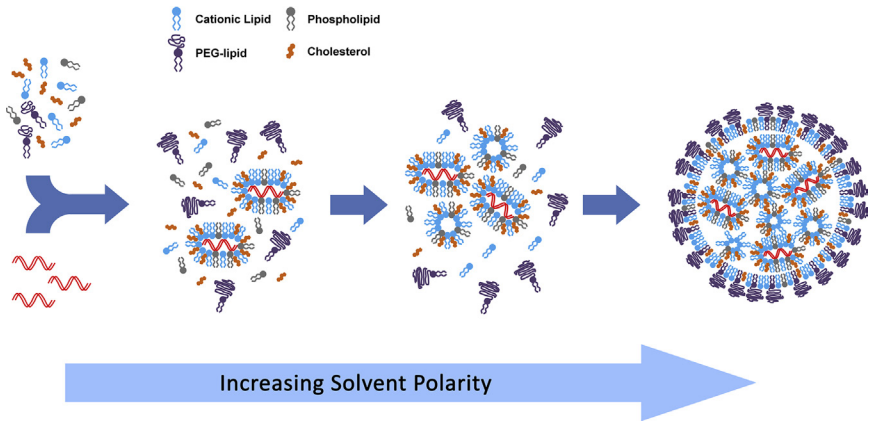


Figure 4.9 ***Assembly of LNP-siRNA by microfluidic mixing.*** Lipid components reach their individual solubility limit and precipitate out of solution as the ethanol in the lipid stream is being diluted by the aqueous stream. The acidic sodium acetate buffer protonates the ionizable cationic lipids (blue), which then form an inverted micelle around the siRNA (red) via electrostatic interaction. As the polarity of the solvent increases, inverted micelles begin to aggregate, which is followed by coating by PEG lipids (purple) to form the LNP-siRNA.

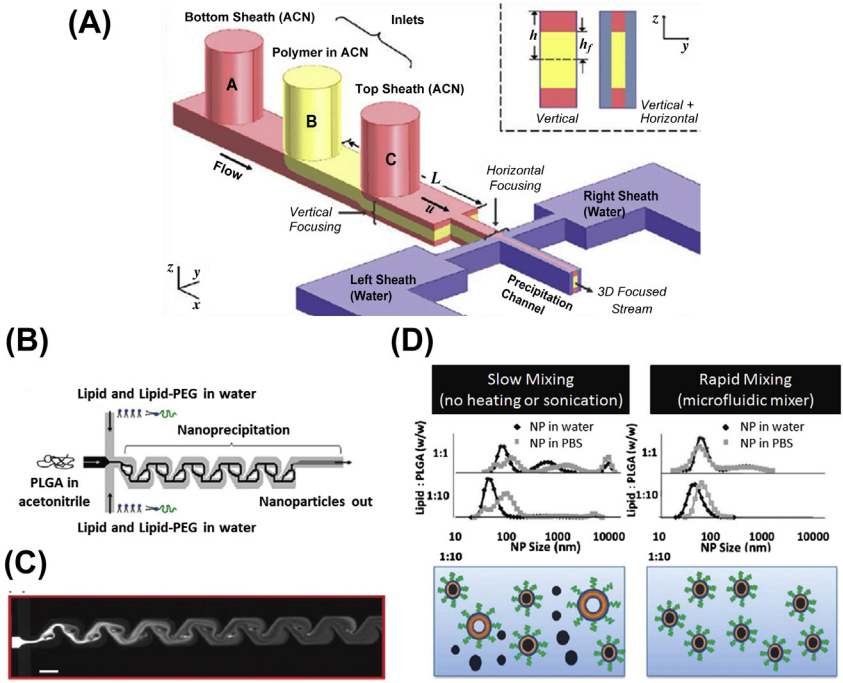


Figure 5.1 (A) Schematic illustration of 3D hydrodynamic focusing composed of three inlets for vertical focusing and separate inlet for side sheath flows. *(Reprinted with permission from Rhee et al. (2011).)* (B) Schematic illustration of the microfluidic device with three inlets that allows the formation of lipid-coated polymeric nanoparticles in the microchannels with Tesla structures. (C) Solvent mixing in the Tesla channels using fluorescent dye and water respectively. The mixing is complete at the fourth turn in the channel. (D) Comparison of slow and rapid mixing of lipid and PLGA solutions. Aggregation forms under slow mixing conditions without the input of any energy, but not under rapid mixing conditions. *(Reprinted with permission from* Valencia et al. (2010)*.)*

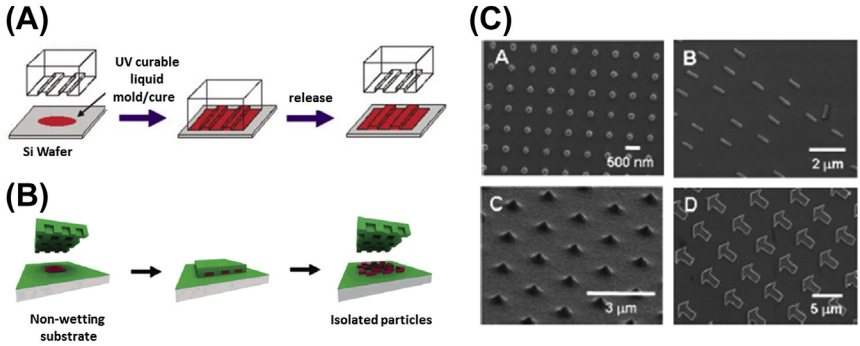


Figure 5.2 (A) Schematic illustration of the imprint lithography process. *(Reprinted with permission from Rolland et al. (2004).)* (B) Schematic illustration of the PRINT process. In PRINT, the nonwetting feature allows the generation of isolated particles. (C) Manipulation of PRINT nanoparticles with different shape and size. *(Reprinted with permission from Rolland et al. (2005).)*

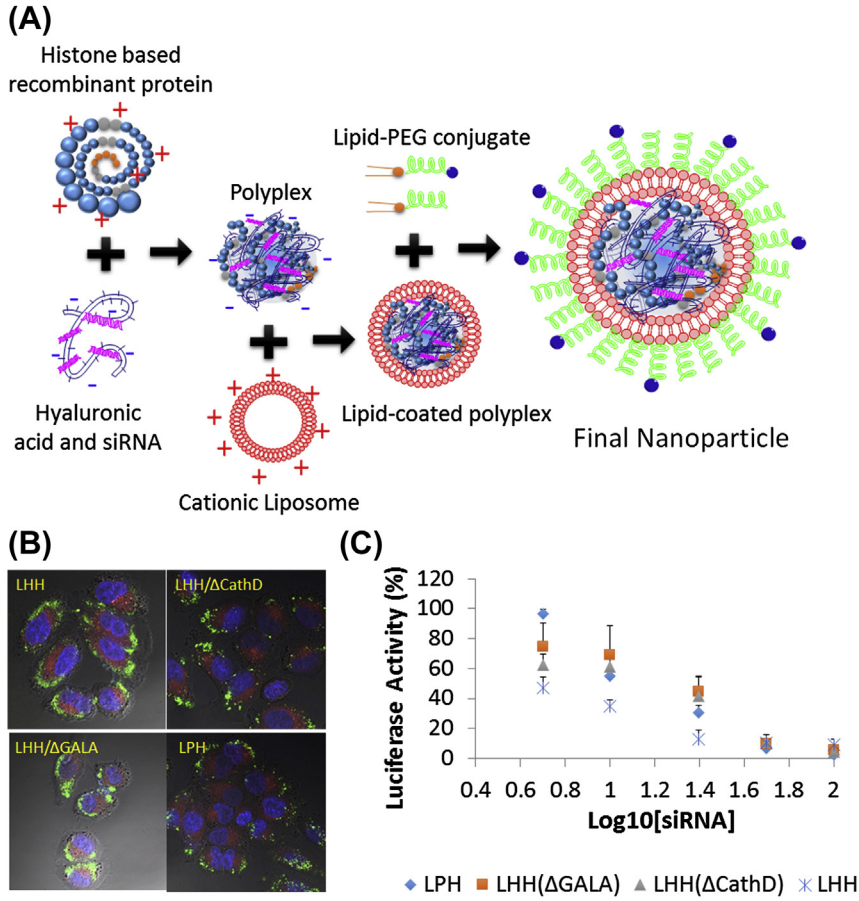


Figure 5.3 (A) Schematic illustration of fabrication of polyplex core-based composite nanoparticle for siRNA delivery using histone-based recombinant protein as nucleic acid condensing agent. (B) Intracellular release profiles of oligonucleotides using the different histone-based recombinant proteins and mutants. Lack of degradation element (LHH/ΔCathD) prohibited the dissociation of oligonucleotides and the punctate forms were observed. (C) IC50 of luciferase knockdown with antiluciferase siRNA delivered by different nanoparticles. *Reprinted with permission from Wang et al. (2013).*

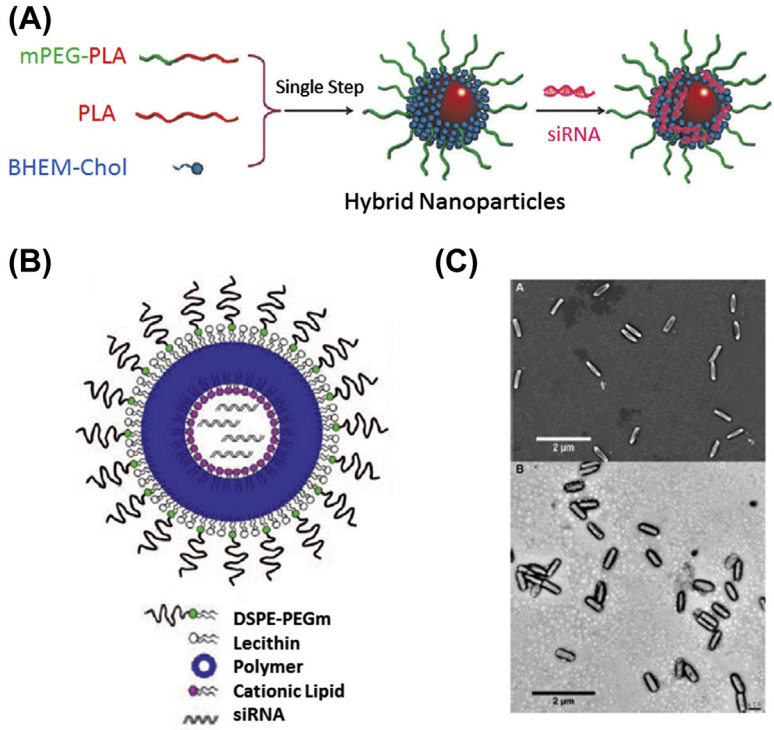


Figure 5.4 (A) Schematic illustration of fabrication of lipid-coated PLA nanoparticles with siRNA adsorbed on the surface of carrier. *(Reprinted with permission from Yang et al. (2012).)* (B) Schematic representation of lipid–polymer nanoparticle composed of outer lipid-PEG, a middle polymer layer, and an inner cationic lipid hollow core for siRNA entrapment. *(Reprinted with permission from Shi et al. (2011).)* (C) SEM (top) and TEM (bottom) images of lipid-coated PRINT nanoparticles composed of PLGA and siRNA. *(Reprinted with permission from Hasan et al. (2012).)*

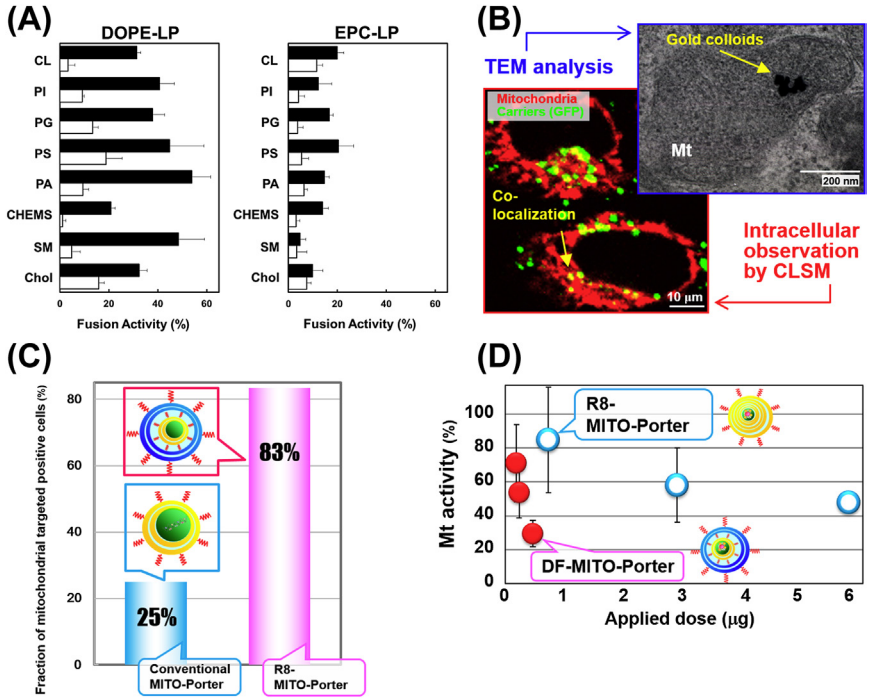


Figure 6.7 ***Summary of experimental results concerning mitochondrial delivery by MITO-Porter system.*** Screening of fusogenic lipid compositions with the mitochondrial membrane (A). Fusion activities (%) of DOPE-LP and EPC-LP were calculated in terms of the reduction of FRET. Closed bars, R8-LP; open bars, unmodified liposome. Data are represented by the mean ± S.D. ($n = 3$). Intracellular observation of the MITO-Porter (green) after staining mitochondria (red) and TEM analysis indicating that Gold colloids encapsulated in the MITO-Porter were delivered to mitochondria (B). The fraction of mitochondrial targeted positive cells was calculated using a confocal image-assisted integrated quantification method (C). Comparison of mitochondrial activity after DNase I delivery between DF-MITO-Porter and R8-MITO-Porter (D). The closed and open circles represent the values corresponding to the mitochondrial activity (%), when the DF-MITO-Porter and the R8-MITO-Porter were used. Data are represented as the mean ± S.D. ($n = 3$–4). *These figures are reproduced from Nakamura et al. (2012), Yamada et al. (2008), Yamada et al. (2011).*

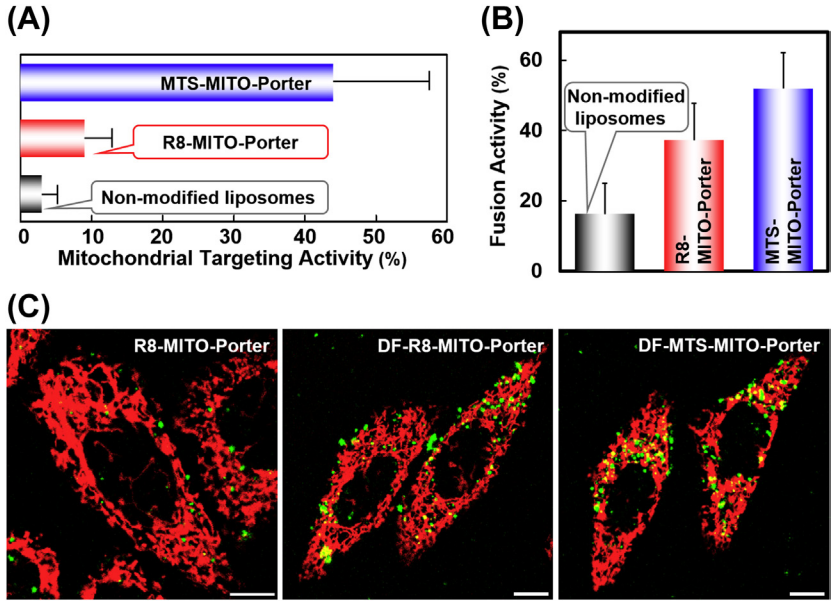


Figure 6.8 ***Evaluation of mitochondrial targeting of MITO-Porter integrated with MTS.*** Mitochondrial targeting activity of the MTS-modified MITO-Porter, R8-modified MITO-Porter, and nonmodified liposomes (A). Mitochondrial membrane fusion activity of the MTS-MITO-Porter, the R8-MITO-Porter, and nonmodified liposomes (B). Data are the means ± S.D. ($n = 3–4$). Intracellular observation of carriers (R8-MITO-Porter, DF-R8-MITO-Porter, DF-MTS-MITO-Porter) (C). The carrier (green) was observed to co-localize with red stained mitochondria, observed as a yellow signal. Scale bars, 10 μm. *These figures are reproduced from Kawamura et al. (2013), Yamada and Harashima (2013).*

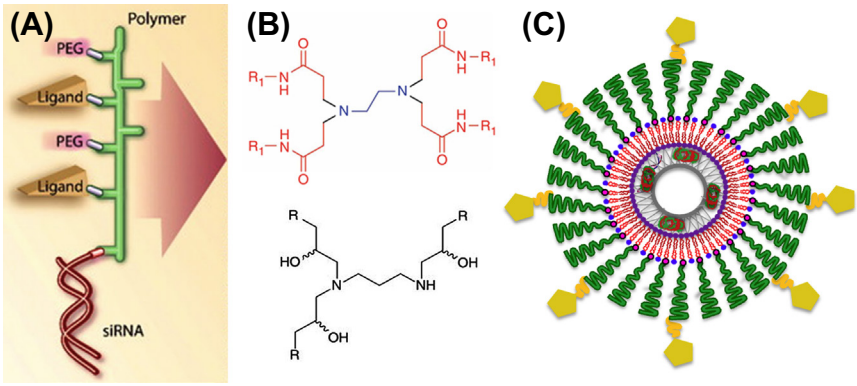


Figure 7.1 ***A sampling of nonviral vectors for gene delivery.*** (A) Dynamic PolyConjugate, employing a grafted design of antifouling molecules, targeting ligands, and therapeutic RNA built off of a PBAVE polymer backbone. (B) Model lipidoids, synthesized via either Michael addition or epoxide chemistry. (C) LCP, a calcium phosphate core supporting an asymmetric, cationic, and PEGylated lipid bilayer, decorated at the surface with targeting ligands. PEG, polyethylene glycol. *Adapted with permission from Akinc et al. (2008), Hu et al. (2013), Rozema et al. (2007), Love et al. (2010).*

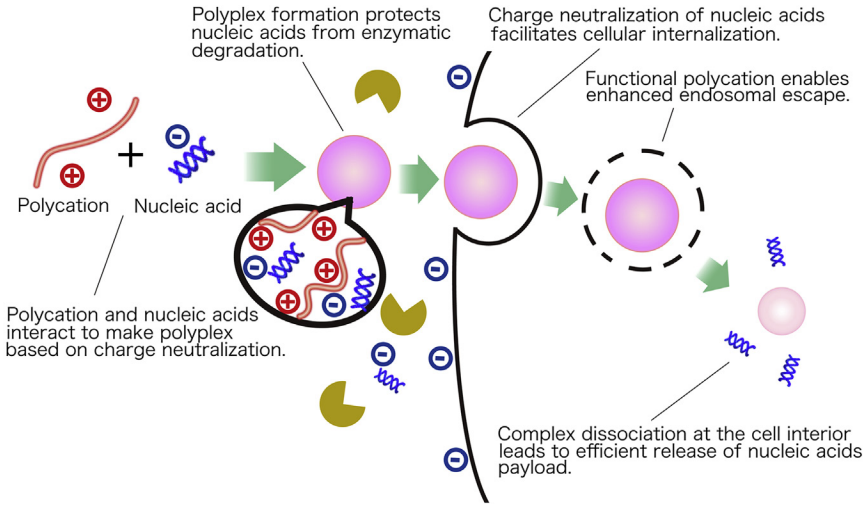


Figure 10.1 ***Strategies for efficient nucleic acids delivery based on polyplex formation.***

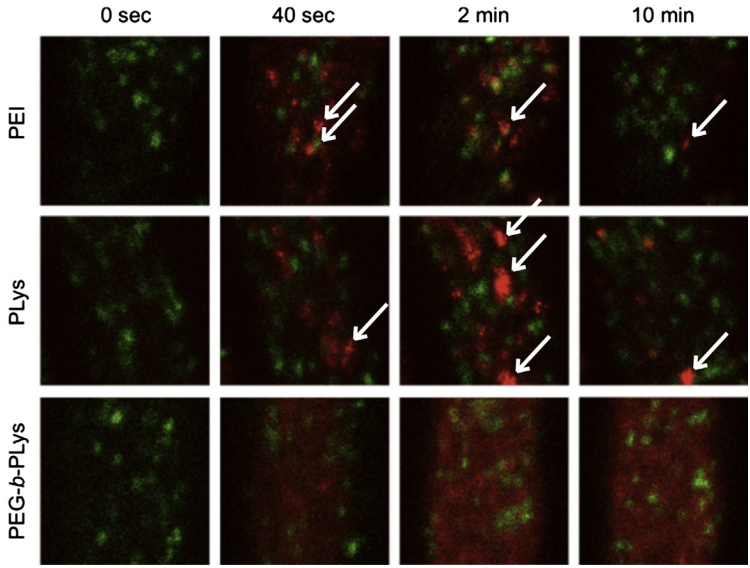
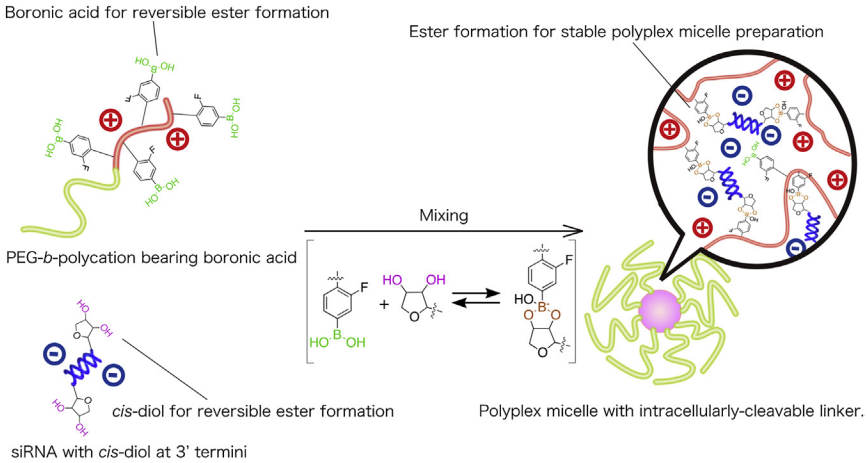


Figure 10.3 ***Representative intravital confocal videographs of homopolymer polyplexes and polyplex micelles circulating in the bloodstream of a mouse earlobe (image size: ~80 × ~80 μm).*** Homopolymer polyplexes were prepared with Cy5-labeled pDNA and PEI (22 kDa) or PLys (4–15 kDa). Polyplex micelles were prepared with Cy5-labeled pDNA and PEG-*b*-PLys (12 kDa for PEG and 7 kDa for PLys). Platelets were stained with DyLight 488-labeled antibody prior to observation. The fluorescence signals of Cy5 and DyLight 488 are shown in red and green, respectively. Arrows indicate the representative microscale aggregates. This figure is reproduced with permission from Nomoto et al. (2011).



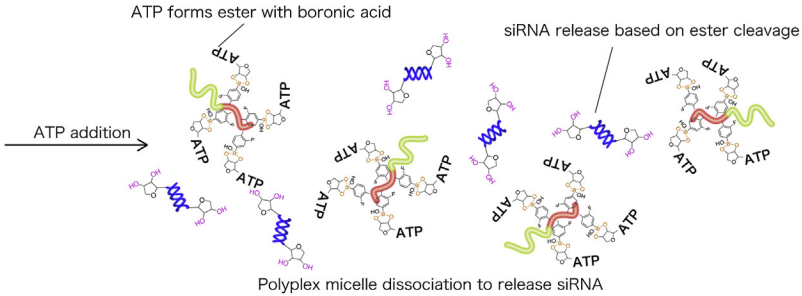


Figure 10.12 ***Strategy of ATP-responsive polyplex micelles.***